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A STUDY, BY MEANS OF PAPER CHROMATOGRAPHY OF THE GROWTH STIMULATING PROPERTIES OF LIVER EXTRACT, YEAST SOLUBLES, AND TRYPSIN DIGEST OF CASEIN FOR *LACTOBACILLUS CASEI*[1]

BY IVAN W. COLEMAN

Abstract

Transverse sections of linear partition chromatograms on filter paper when eluted with basal medium for *Lactobacillus casei* showed Wilson Liver Fraction L to contain at least two growth stimulating factors for this organism. Three growth stimulating factors were demonstrable by this technique in trypsin digest of casein in contrast to dried yeast solubles which contained but one such factor. The growth stimulating factor from yeast was separated and partially purified. Treatment with strong acid suggested the peptide nature of this material. Chromatographic evidence of the presence of glycine, leucine, aspartic acid, and glutamic acid was obtained from the acid hydrolyzate of the yeast factor.

Introduction

When *Lactobacillus casei* is grown at 37° C. in a synthetic medium containing all the known growth essentials, maximal growth is obtained only with 36-72 hr. incubation. Sprince and Woolley (6, 7) observed that the addition of Wilson Liver Fraction L or tryptic digest of casein stimulated growth such that maximal growth could be obtained in 16-20 hr. A like stimulatory effect was observed later by Scott, Norris, and Heuser (5) with dried brewer's yeast. Woolley attributed the stimulation to unknown peptide factor or factors contained in these materials to which he applied the name strepogenin. Attempts to isolate strepogenin succeeded only in achieving concentrates of high potency but this work established the high solubility of the factors in phenol and water. This property suggested the possibility of purifying the crude sources of the factors by partition between phenol and water using the technique of partition chromatography advanced by Consden, Gordon, and Martin (1).

This paper reports some observations made on the growth stimulating properties of three sources of strepogenin after fractionation of the mixtures had been accomplished by the chromatographic technique.

[1] *Manuscript received March 15, 1951.*

A contribution from the Department of Biochemistry, Faculty of Medicine, University of Manitoba, Winnipeg, Man.

Materials

Lactobacillus casei No. 7461 was obtained from the American Type Culture Collection. Stab cultures were carried in an aqueous medium consisting of peptone 1%, glucose 1%, sodium carbonate 0.5%, and agar 1%. Transfers were made weekly to a broth of the same composition but lacking agar. The subcultures were incubated at 37° C. for 24 hr. and returned to agar stabs for storage at 5° C. One day prior to an assay, an inoculum consisting of 10 ml. of basal medium, 20 mgm. of Difco dried yeast solubles, and 20 mgm. of Liver fraction L was inoculated from an agar stab and incubated for 18 hr. at 37° C. The cells were centrifuged free from inoculum and washed by suspending them three times in sterile saline (0.85%). They were diluted for assay tube inoculation with saline to yield a galvanometer deflection of 95 on an Evelyn colorimeter fitted with a 520 mμ filter.

The medium used in this work is shown in Table I as prepared at double the concentration used. The assays were run in clean Evelyn tubes. Basal medium (5.0 ml.) was added and the volume made up to 10.0 ml. with glass

TABLE I

COMPOSITION OF BASAL MEDIUM

Glucose	20 gm.	Biotin	0.8 μgm.
Sodium acetate (anhydrous)	12 gm.	Folic acid	0.04 μgm.
Casein hydrolyzate (HCl)	10 gm.	Manganese chloride $4H_2O$	20 mgm.
Cystine	0.4 gm.	Salt solution A	20 ml.
l-Tryptophane	0.1 gm.	Salt solution B	20 ml.
Adenine sulphate	20 mgm.	pH adjusted to 6.8	
Guanine hydrochloride	20 mgm.	Glass distilled water	to 1000 ml.
Uracil	20 mgm.	Salt solution A	
Xanthine	20 mgm.	K_2HPO_4	25 gm.
Riboflavin	400 μgm.	KH_2PO_4	25 gm.
Thiamine hydrochloride	200 μgm.	Water	500 ml.
d-Calcium pantothenate	200 μgm.	Salt solution B	
Pyridoxine hydrochloride	200 μgm.	$MgSO_4 . 7H_2O$	10.0 gm.
p-Aminobenzoic acid	200 μgm.	$FeSO_4 . 7H_2O$	0.5 gm.
Nicotinic acid	200 μgm.	$MnSO_4 . 4H_2O$	0.5 gm.
		Water	500 ml.

distilled water. After the tubes were plugged with cotton, they were sterilized by steaming for 15 min. This procedure was used to avoid discoloring the media with decomposition products which occurred on autoclaving.

The assay tubes were inoculated with one drop of dilute saline suspension using a vertically mounted sterile syringe-barrel fitted with a No. 22 needle. This simple assembly produced a uniform sized drop at a rate of 10 drops per minute. This rate was sufficiently slow that one drop could be placed in each tube of medium without touching the sides of the tube. The inoculated tubes were incubated for 18 hr. at 37° C. After uniform shaking, growth in each tube was measured turbidimetrically using the Evelyn colorimeter fitted with a 520 mμ filter.

Casein hydrolyzate for media was prepared by hydrolyzing "vitamin-free" casein (Nutritional Biochemicals) with 5*N* hydrochloric acid for 72 hr. under continuous reflux. The acid was removed by repeated distillations and the residue neutralized with dilute sodium hydroxide. This solution was cleared by stirring with activated charcoal (Norit autoclaved three hours at 20 lb. pressure) and filtered through Filter-Cel. Casein hydrolyzate to be used as a source of strepogenin was prepared by digesting crude casein with Difco trypsin (activity 1 : 110) for 30 hr. at 37° C. This digest was purified by precipitation with basic lead acetate and the removal of the excess lead by hydrogen sulphide. After boiling to remove hydrogen sulphide the resulting solution was evaporated to dryness under reduced pressure and dried in a vacuum oven at 60° C.

Methods

Chromatography

Filter paper (Schleicher and Schuel No. 595) was cut into sheets 10 in. × 18 in. and marked with a base line 2¼ in. from one end. A 10 mgm. quantity of the material to be fractioned was placed in a strip not exceeding 5 mm. in width along this base line and dried in a warm air stream. A fold was made in the paper approximately 1¾ in. from the end and the paper placed in the solvent trough. Paper and trough were then placed in a gastight box of paraffin coated plywood similar to the chromatograph chambers of Dent for chromatography by downward movement of solvent (3). The chambers were maintained at 21 ± 1.0° C. in a controlled temperature cabinet. The saturated phenol solvent was prepared by liquefying phenol crystals (B.D.H.) and then equilibrating by shaking with an equal volume of water at 21° C. The phenol-poor phase was placed in a shallow glass container at the bottom of the chromatography chamber and the sheets were allowed to become saturated with the vapor of this solution for four hours before the phenol-rich layer was placed in the trough. The solvent was allowed to move downward for 30–35 cm. (16–18 hr.) at which time these sheets were removed and dried in a current of warm air. Although very little discoloration occurred, occasional stains were removed before further use. A test strip 1 in. wide was cut longitudinally and developed with ninhydrin. If fractionation was such that 20–25 distinct zones could be seen on the test strip, the remainder of the chromatogram was sectioned in 20 strips cut at right angles to the direction of the movement of the solvent and parallel to the solvent front. These strips were freed from phenol by ether extraction in an offset Soxhlet extractor until no reaction was given by Folin's and Ciocalteau's phenol reagent (4). Ether was removed from the strips by heating on a water bath. The strips were placed in Evelyn tubes, 5.0 ml. of concentrated basal media were added, and the tubes shaken for one hour. The filter paper strip was then removed, squeezed dry with a small spatula, and washed with 1–2 ml. water. The media and washings were then made up to a volume of 10.0 ml. with water. Sterilization by steaming and inoculation followed in the manner previously

described. The tubes were incubated for 18 hr. at 37° C. and the growth determined turbidimetrically. Cultures containing graded amounts of Difco dried yeast solubles were prepared simultaneously as standards so that growth in the tubes containing eluates from the chromatogram strips could be compared with the turbidity of the standard series. Uninoculated control duplicates of each chromatograph strip were included in order to check for sterility but with the sterile precautions taken, no contamination resulted.

When the "collidine" solvent was used, a mixture of collidine, lutidine, and water (1 : 1 : 2 by volume) was prepared and equilibrated in the same manner as with phenol. Subsequent removal of collidine before assay was done by ether extraction as before but the time of extraction was increased to 24 hr.

Results and Discussion

Chromatographic Separations in Phenol

Chromatograms of Difco dried yeast solubles were prepared in duplicate. One was sectioned to determine the R_f of the stimulating zones; the other was retained for later chemical examination of the active zone. The results of growth studies on three sectioned chromatograms of yeast are shown in Fig. 1

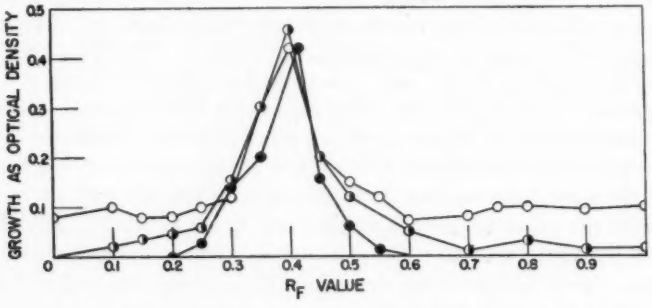

FIG. 1. The growth of *L. casei* with successive sections of phenol chromatograms of Difco dried yeast solubles. The results of three separate runs are shown.

in which growth of *L. casei* with basal medium elution of each successive strip from base line to solvent front is plotted as the optical density against the R_f value of the strip. Only one zone of stimulation was observed occurring at an R_f value of 0.41, the value of the "peak" of the curve. This interpretation is based on the criteria advanced by Consden *et al.* (1) and Craig (2). These authors demonstrated that in a countercurrent separation, the distribution of a substance throughout the sequence of extractions was one of a normal frequency distribution and hence showed a symmetrical distribution about one maximal value. The position of this maximal value in the extraction sequence is a function of the partition coefficient. If two or more materials having

different partition coefficients are distributed, then each distributes as a normal frequency distribution. These distributions will tend to merge but as long as the difference in their partition coefficients exceeds the resolving power of the extraction sequence, the "peaks" will remain distinct and each will be representative of a separate material. In the examination of the yeast chromatograms, it appears probable that the growth of *L. casei* is proportional to the concentration of growth stimulant in each strip. The plot of this growth against the R_f value of the strip is thus a measure of the distribution of this growth stimulant throughout the extraction train. Thus the number of "peaks" can be taken as indicative of the minimum number of growth stimulants present in the original mixture and the identity of each growth stimulant related to the R_f value of each peak.

Chromatograms of Liver Fraction L in phenol were similarly examined.* With this material, growth of *L. casei* in sections of the chromatogram along the line of elution is shown in Fig. 2. Two distinct peaks in these curves were

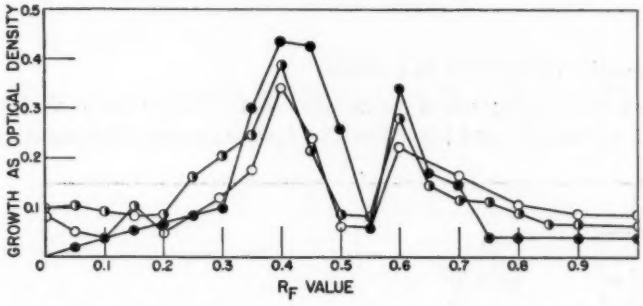

FIG. 2. The growth of *L. casei* with successive sections of phenol chromatograms of Wilson Liver Fraction L. The results of three separate runs are shown.

observed at R_f values of 0.40 and 0.60 respectively. From these curves it was concluded that Liver Fraction L contains at least two growth stimulating factors for *L. casei*.

The results of similar studies on trypsin hydrolyzate of casein are shown in Fig. 3. These curves show at least three significant peaks at R_f values of 0.15, 0.42, and 0.70 from which it was concluded that casein contains at least three chemically distinct growth stimulating factors for *L. casei*.

The occurrence of the factor of R_f value, 0.40 in each of these strepogenin sources suggests the possibility of the same factor in each material. This may prove to be the case but cannot be established on the chromatographic

* *Attempts were also made to fractionate antipernicious anemia extracts of liver by partition chromatography. These attempts were not successful but the author wishes to thank Dr. A. Lee Caldwell of the Eli Lilly Co. for a generous gift of this material.*

evidence alone. Compounds of widely different composition may occupy the same position in a partition chromatogram. This is the case with valine, tyrosine, and methionine in a phenol chromatogram.

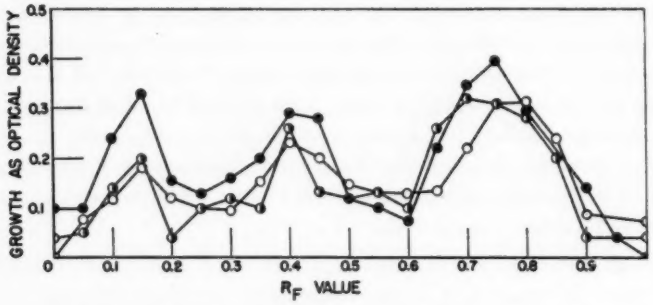

FIG. 3. The growth of *L. casei* with successive sections of phenol chromatograms of trypsin digest of casein. The results of three separate runs are shown.

Chromatographic Separations in Collidine

Fig. 4 is a composite graph of the growth obtained when assays of chromatograms of yeast solubles and Liver Fraction L were carried out separately using

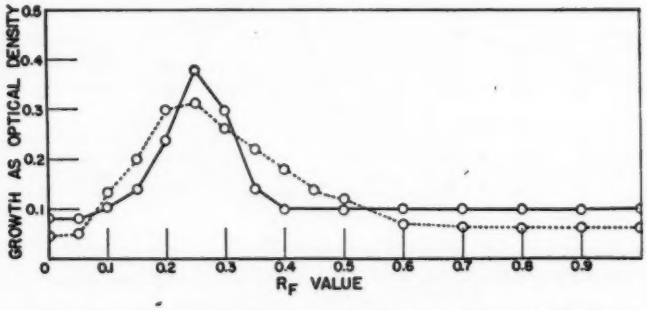

FIG. 4. The growth of *L. casei* is plotted at the optical density against the R_f value of sections of collidine chromatograms of Difco dried yeast solubles (solid line) and Liver Fraction L (dotted line).

collidine as the organic solvent. Both these materials show one zone of stimulation at an R_f value of 0.25. The two growth stimulating zones demonstrable with phenol elution of Liver Fraction L merged, when collidine was used, to form one single broad zone.

It was not found possible to fractionate the trypsin digest of casein using collidine as the solvent. Each attempt resulted in chromatograms showing undifferentiated striation from base line to solvent front.

Hydrolysis of Yeast Factor

Duplicate chromatograms of dried yeast solubles from phenol separation were sectioned to remove the zone from R_f 0.40 to 0.45. This strip was macerated in 5.0 ml. water and the filter paper removed by filtration through a sintered glass funnel. The solution was reduced to dryness and the remaining solids taken up in 50 μl. of water. Ten microliters of this solution was then chromatographed in collidine to determine the complexity of the zone. To the remaining solution was then added 2.0 ml. of 5*N* hydrochloric acid and the whole washed into a Pyrex test tube and sealed. The assembly was then placed in an oven at 100° C. for 24 hr. A sample of this hydrolyzate was taken and the hydrochloric acid was removed by distillation under reduced pressure. The solids were taken up in 2.0 ml. water and again evaporated to dryness. Forty microliters of water was then added and transferred to a chromatogram sheet for fractionation in collidine. The remaining hydrolyzate was replaced in the oven for an additional 48 hr. and the procedure for acid removal repeated. Amino acid standards were run simultaneously. The results of this chromatographic analysis are shown in Table II.

TABLE II

THE R_f VALUES IN COLLIDINE OF THE COMPONENTS OF THE YEAST FACTOR BEFORE AND AFTER HYDROLYSIS

	R_f Value						
Before hydrolysis	0.06				0.25		
With 24 hr. hydrolysis	0.06	0.13	0.17	0.20	—	—	0.57
With 72 hr. hydrolysis	0.06	—	0.17	0.21	—	0.33	0.57
Amino acids	None		Aspartic acid	Glutamic acid		Glycine	Leucine

The collidine chromatogram of the Yeast Factor before hydrolysis showed two ninhydrin reacting spots at 0.06 and 0.25. However, it had been previously shown (See Fig. 4) that only the 0.25 zone was active. After 24 hr. hydrolysis four zones were detectable in addition to the retained inactive zone at R_f 0.06. After 72 hr. the zone at R_f 0.13 disappeared and a new zone at R_f 0.33 appeared. The four zones showed R_f values similar to those of aspartic acid, glutamic acid, glycine, and leucine, respectively, and occupied the same positions on the chromatograms as those amino acids run simultaneously as controls.

These results appear to indicate that the growth-stimulating material occupying the R_f position of 0.25 in the collidine separation was a complex substance, fissionable by hydrolysis in strong acid, and possibly a polypeptide.

This substance yielded on 72-hr. hydrolysis four compounds which could not be distinguished in the technique used from aspartic acid, glutamic acid, glycine, and leucine.

This substance appeared somewhat resistant to hydrolysis. On 24 hr. hydrolysis a zone was distinguishable which on 72 hr. hydrolysis completely disappeared. Coincident with the disappearance of this zone, a new zone appeared at an R_f similar to that of glycine which would suggest that the intermediate zone respresented that of a glycine containing compound.

Evidence indicating the peptide nature of the factor present in yeast is in agreement with the postulation of the peptide nature of "strepogenin" advanced by Woolley (8). However, the substance involved can bear little resemblance to the active peptides synthesized by this author (seryl-glycylglutamic and glycylserylglutamic). No evidence could be obtained from the acid hydrolyzate of the yeast factor which would indicate the presence of serine shown by this same author to be an essential component of the active peptides.

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EFFECT OF SODIUM KEMITHAL, TRIMETHADIONE, AND SODIUM DIPHENYLHYDANTOINATE ON SKELETAL MUSCLE[1]

By Bernard E. Riedel and Mervyn J. Huston

Abstract

Sodium kemithal, 325 mgm. per kgm., injected intraperitoneally into rats caused a marked depression in the response of normal and denervated striated muscle. The action is believed to be directly on the muscle. Trimethadione produced a sudden marked depression at an intraperitoneal dosage level of 3500 mgm. per kgm. of both normal and denervated muscles. This action would appear to be due to a direct muscular effect. Sodium diphenylhydantoirate produced no significant effect on the gastrocnemius muscle at a dose of 250 mgm. per kgm. by intraperitoneal injection.

Introduction

In previous communications it was shown that sodium amytal, at a dose of 190 mgm. per kgm., had a transient stimulant effect on muscle (4), and at 350 mgm. per kgm. a marked depressant effect (5). It was thought of interest to test, in a similar manner, the effect of certain other depressant drugs on the somatic system of the rat. The drugs used in the following experiments were: an ultra-short-acting barbiturate, sodium kemithal; and two anticonvulsants, trimethadione (Tridione-Abbott) and sodium diphenylhydantoinate (Diphenate-C. Frosst and Company).

Method

The experimental method was the same as that previously described (4).

Results

Sodium Kemithal

When 325 mgm. per kgm. of sodium kemithal was injected intraperitoneally into 10 rats, five showed a slight initial increase of muscle response but the mean was not significantly raised. After three minutes the mean showed a progressive decrease which continued over the experimental period. Two experiments showed no decrease. In the case of those legs which showed a decrease no recovery was noted up to periods of several hours. Although there was considerable variation from animal to animal, it would appear that the depressant action is significant when the mean is compared to the mean of 30 control animals (Table I and Fig. 1).

Similar results were obtained when animals were used whose legs had been denervated for five days. A slight initial increase was observed in 7 of 10 experiments but the mean was not significantly raised. The subsequent

[1] *Manuscript received in original form March 20, 1951, and, as revised, April 12, 1951. Contribution from the School of Pharmacy, University of Alberta, Edmonton, Alta.*

TABLE I

EFFECT OF SODIUM KEMITHAL, TRIMETHADIONE, AND SODIUM DIPHENYLHYDANTOINATE ON THE GASTROCNEMIUS MUSCLE OF THE RAT

Drug	Dose, mgm. per kgm.	State of muscle	Mean % of initial contraction tension at 72 min.	Mean control at that time	Difference
Sodium kemithal	325	Normal	59	91	32
Sodium kemithal	325	Denervated	54	85	31
Trimethadione	3500	Normal	69	91	22
Trimethadione	3500	Denervated	47	85	38
Sodium diphenylhydantoinate	250	Normal	83	91	8

NOTE: *The number of animals used in each case was 10 experimental and 30 control.*

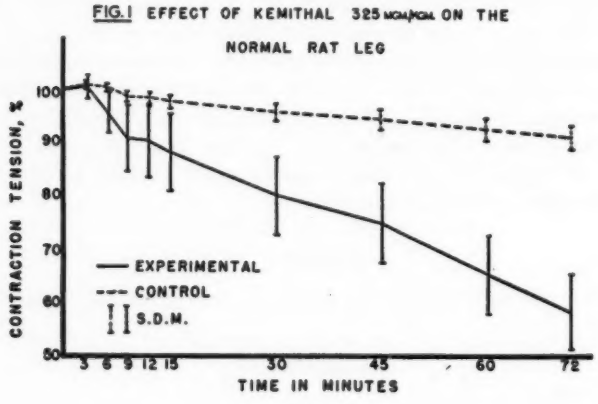

FIG. 1.

depressant action is apparently significant (Table I and Fig. 2). When the experimental results on normal and denervated muscle are compared relative to their respective controls there is remarkably little difference (Table I and Fig. 3). It would appear, therefore, that the depressant action is primarily a direct one on the muscle and not on the neuromyal junction. The effect of sodium kemithal, 325 mgm. per kgm., is similar to that seen with high doses of sodium amytal (5).

Trimethadione (Tridione-Abbott)

Since the acute toxicity of trimethadione is remarkably low (3) it was possible to give relatively enormous doses without early fatality. A dose of 3500 mgm. per kgm. was used by intraperitoneal injection. The animals were deeply depressed and died after a period of several hours.

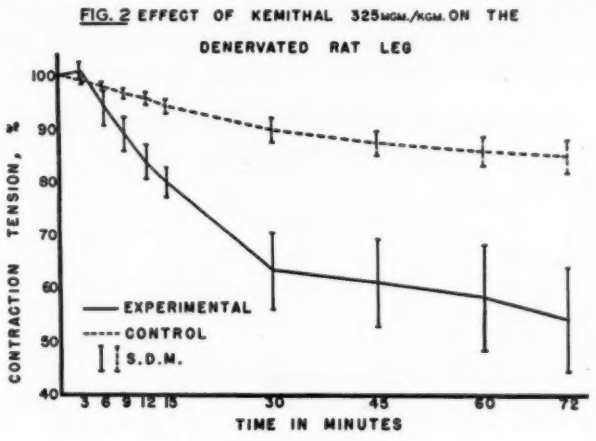

FIG. 2.

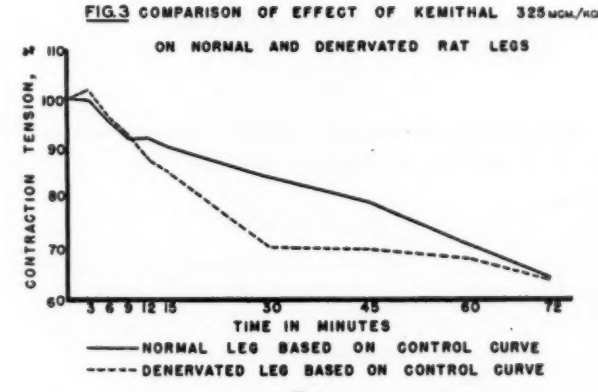
FIG. 3 COMPARISON OF EFFECT OF KEMITHAL 325 MGM./KGM. ON NORMAL AND DENERVATED RAT LEGS
CONTRACTION TENSION, %
110
100
90
80
70
60
3 6 9 12 15 30 45 60 72
TIME IN MINUTES
NORMAL LEG BASED ON CONTROL CURVE
DENERVATED LEG BASED ON CONTROL CURVE

FIG. 3.

The results obtained in 10 experiments showed a sudden marked decrease of response with little variation from animal to animal. No significant recovery was obtained (Fig. 4).

Similar results were obtained with denervated muscle (Fig. 5). When comparison of the effect on normal and denervated muscle is made relative to their respective controls it is seen that the action is essentially the same (Fig. 6). It therefore appears that the action of trimethadione is directly on the muscle (Table I).

Sodium Diphenylhydantoinate (Diphenate, Frosst)

A series of 10 experiments was run using 250 mgm. per kgm. of sodium diphenylhydantoinate injected intraperitoneally. Varied results were obtained. Six experiments showed a slight increase of response to a maximum

mean of 104% at 12 min. The variation was so wide that this result cannot be considered statistically significant. The same is true of the subsequent slight decrease of response (Table I).

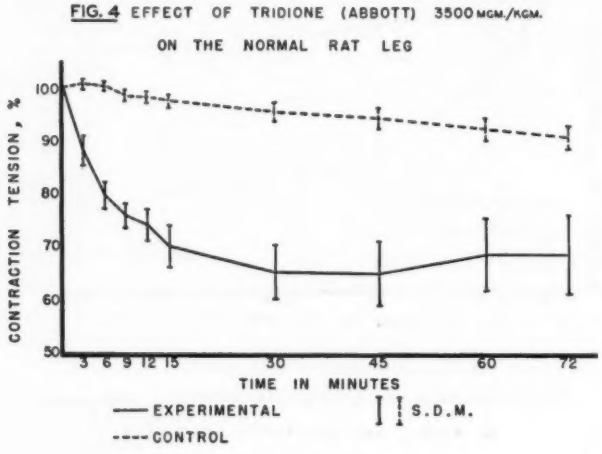

FIG. 4.

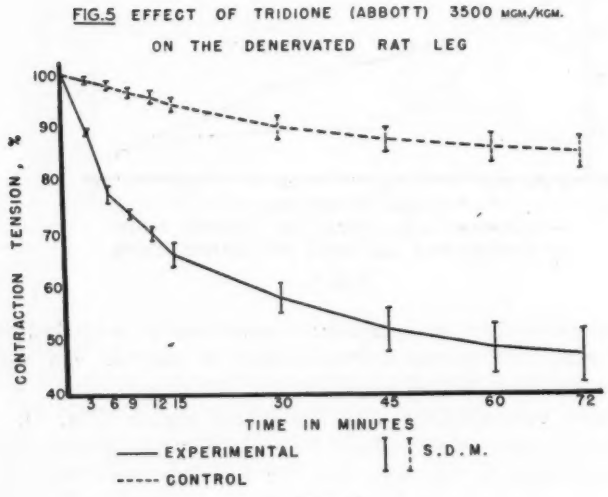

FIG. 5.

Similar variations in response have been shown in the central effects of sodium diphenylhydantoinate. Intraperitoneal injection in rats of a dose just under the LD50 was shown by Gruber and co-workers (2) to cause excitement, followed by exophthalmus, unsteady gait with the posterior

extremities spastic and spread far apart in walking, and finally the animal either went into convulsions or became very depressed. It has also been shown that as an anticonvulsant single large doses of the drug administered parenterally

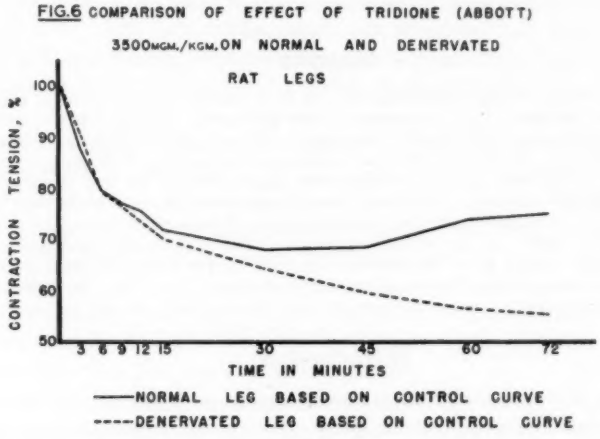

FIG. 6.

are not protective against convulsions induced by convulsant drugs but even appear to act synergistically with metrazol to produce long lasting severe convulsions (1).

Acknowledgments

The sodium kemithal used was supplied by Imperial Chemicals Limited; the Tridione by Abbott; and the Diphenate by C. Frosst and Company.

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EFFET DE L'ACIDE ASCORBIQUE SUR L'HYPERTENSION EXPÉRIMENTALE[1]

PAR O. HÉROUX ET L.-PAUL DUGAL

Abstract

In previously reported observations, we have shown that (*a*) ascorbic acid, given in large doses, had a hypotensive effect on normal blood pressure, (*b*) the initial hypotension, due to sudden exposure to cold, was prevented by large doses of ascorbic acid, (*c*) the hypertension encountered upon long exposures to cold was lowered by ascorbic acid. The present paper confirms the last result and also shows that actually ascorbic acid has a hypotensive action on experimental hypertension obtained by the two following ways: (1) excess of sodium chloride in drinking water and (2) renal compression. The hypertension and adrenal hypertrophy caused by a long exposure to cold regress when the animals are brought back to normal temperature. In its initial phase, the experimental renal hypertension is lowered by ascorbic acid but not in its chronic phase.

Introduction

Au cours de nos recherches antérieures, nous avons montré: (1) que l'acide ascorbique donné à forte dose avait un effet hypotenseur marqué chez l'animal normal (18); (2) qu'il prévenait l'hypotension normalement observée dans les cas d'exposition brusque au froid et (3) qu'il ramenait à la normale l'hypertension provoquée par une longue exposition au froid (18).

Mais comme ce dernier résultat avait été obtenu sur un petit nombre d'animaux, nous avons décidé de reprendre cette expérience.

Davis et Poser (2) en 1943 avaient déjà observé que l'administration quotidienne d'un gramme d'acide ascorbique, à un petit nombre de leurs patients souffrant d'hypertension, avait amené une amélioration clinique objective ou subjective et même les deux chez la plupart d'entre eux. Mais un peu plus tard, Wakerlin (19) rapporta qu'il n'avait observé aucun effet bienfaisant chez un chien hypertendu qu'il traita avec de fortes doses d'acide ascorbique.

On admet maintenant que les surrénales jouent un rôle essentiel dans l'hypertension (11, 12, 14, 17). Les travaux de Dugal *et al.* (3, 4, 5, 18) ayant montré que l'acide ascorbique augmente l'activité des surrénales, nous nous sommes crus justifiables de penser, malgré le résultat de Wakerlin obtenu d'ailleurs sur un seul sujet, que l'acide ascorbique pouvait influencer l'hypertension expérimentale dans tous les cas où les surrénales y jouent un rôle primordial; nous avons donc eu recours au froid (15), à la compression rénale et finalement à une alimentation riche en NaCl (1, 13) pour produire l'hypertension expérimentale, et nous avons étudié l'effet de l'acide ascorbique dans chaque cas.

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(1). Hypertension due à une longue exposition au froid

(A) Effet de l'injection d'acide ascorbique

Protocole

Nous avons placé dans une chambre froide maintenue à une température moyenne de 0° C., 126 rats mâles pesant en moyenne 300 grammes. Ils étaient gardés dans des cages séparées et nourris au Purina et à l'eau du robinet. L'eau de boisson était régulièrement changée de façon à éviter la formation de glace.

Toutes les semaines nous avons mesuré leur pression sans les endormir, en nous servant de la méthode pléthysmographique de Friedman (6). Cette méthode, bien qu'assez souvent critiquée, nous est apparue suffisamment sensible pour notre travail. Par des mesures répétées pendant plusieurs jours sur les mêmes animaux, nous nous sommes rendus compte de la validité et de la précision de cette méthode; d'une fois à l'autre et dans les mêmes conditions, nous obtenions sensiblement les mêmes valeurs sur les mêmes animaux. Dès qu'un animal manifestait une élévation de pression d'au moins 40 mm. Hg, nous nous en servions pour faire notre expérience.

Des 126 rats du début, il nous en est resté 27 dont la queue n'était pas nécrosée et qui étaient devenus hypertendus. Parmi ces 27 rats, cinq ont reçu par la bouche tous les jours, durant leur exposition au froid, 150 mg. d'acide ascorbique sous forme d'ascorbate de Na; ces cinq rats forment notre groupe III.

Les 22 autres ont été divisés en deux groupes. Le groupe I en comprend 16 et le groupe II, six.

Une fois l'hypertension de ces rats bien établie, nous avons donné au groupe I, 50 mg. d'acide ascorbique (0.5 cc.) sous forme d'ascorbate de Na, par injection intrapéritonéale, au groupe II, 0.5 cc. d'une solution de $NaHCO_3$ contenant autant de Na que les 0.5 cc. d'ascorbate de Na, et au groupe III, 150 mg. d'acide ascorbique, sous forme d'ascorbate de sodium, toujours par injection intrapéritonéale.

La pression était mesurée immédiatement avant l'injection et une heure après.

Nous avons fait un examen histologique des reins de plusieurs des animaux sacrifiés à différents intervalles: ces reins étaient fixés au Bouin, sectionnés à 6 μ et colorés à l'Hémalun érythrosine safran.

Résultats

Nous avons constaté que la pression artérielle baissait de 27% en moyenne chez les rats traités à l'acide ascorbique mais qu'elle demeurait invariable chez les témoins traités au $NaHCO_3$, de même d'ailleurs que chez ceux qui avaient reçu 150 mg. d'acide ascorbique par la bouche, tout le temps de leur exposition au froid.

Quant aux coupes histologiques des reins que nous avons examinés, elles ne nous ont révélé aucune lésion.

Le fait qu'il n'y ait pas de lésion rénale chez ces hypertendus porte à croire que l'hypertension provoquée par une exposition prolongée à un stress comme le froid est bien plus physiologique que pathologique. Pour sa part, Selye l'avait attribuée à une hypersécrétion du cortex surrénalien (15).

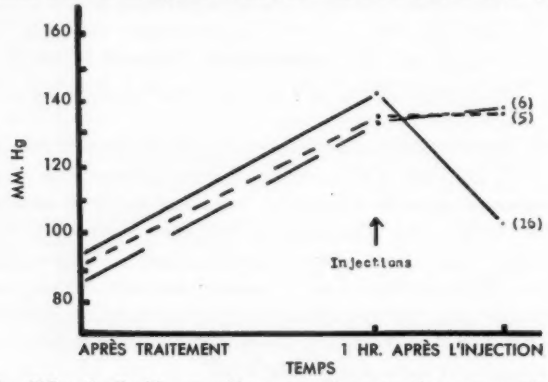

FIG. 1. Effet de l'acide ascorbique sur l'hypertension provoquée par une longue exposition au froid.
Entre parenthèses: nombre d'animaux. ——— = Injection de 50 mg. d'acide ascorbique. — — — — = Injection de 50 mg. d'acide ascorbique (rats gavés en acide ascorbique pendant 2—3½ mois). —— —— = Injection de 0.5 cc. de $NaHCO_3$.

(B) NATURE DE CETTE HYPERTENSION

Protocole

Si réellement l'hypertension provoquée par une résistance prolongée au froid était due à une hypersécrétion glandulaire sans lésions rénales, elle devait disparaître avec la cause responsable du stress, c'est-à-dire avec le retour des rats à température normale.

Pour réaliser cette expérience, nous avons exposé 54 rats d'un poids moyen de 300 grammes à un froid de −2° C. pendant deux mois. Ces animaux étaient gardés dans des cages individuelles, mangeaient du Purina "Fox-Chow" et buvaient de l'eau du robinet ad libitum.

Après deux mois d'expérimentation, trente avaient survécu. A ce moment nous en avons sacrifié onze et nous avons pesé leurs surrénales. Les dix-neuf autres furent ramenés à la température normale et un mois plus tard nous pesions leurs surrénales après les avoir sacrifiés.

Les modifications de la pression ont été étudiées sur huit seulement des 19 rats ramenés à la température normale. Tous les autres avaient une queue trop nécrosée pour permettre une étude plus complète.

Résultats

Voici résumés dans la Fig. 2 et le Tableau I les résultats obtenus sur le poids des surrénales et la pression des hypertendus.

Conclusions

Les résultats obtenus sont donc les suivants:

(1) retour à une pression normale;

(2) régression dans l'hypertrophie des surrénales.

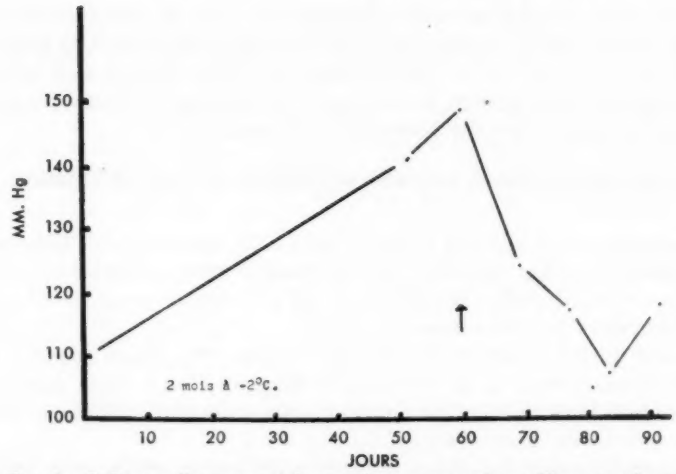

FIG. 2. Influence d'un retour à la température normale sur l'hypertension causée par une exposition de deux mois à −2° C. ↑ = Ramenés à la température normale.

TABLEAU I

INFLUENCE D'UN RETOUR À LA TEMPÉRATURE NORMALE SUR L'HYPERTROPHIE SURRÉNALIENNE ET L'HYPERTENSION PRODUITE PAR UNE EXPOSITION DE DEUX MOIS À 1—2° C.

A. Surrénales

Traitement	Nombre d'animaux	Poids initial	Poids final	Surrénales, mgm.	Diff.	*t*
Rats exposés 2 mois à −2° C.	11	294	265	44.43 ± 3.5	−10.6	2.8
Rats exposés 2 mois à −2° C. et ensuite 1 mois à la température normale	19	301.0	333.8	33.8 ± 1.4		

B. Pression

	Date	Pressions	Différence	*t*
Entrée au froid:	29/6	110 ± 5.0		
	20/8	142 ± 4.0	+ 38.7	5.3
Sortie du froid:	29/8	148.7 ± 5.4		
1 sem. après	5/9	124 ±		
2 sem. après	13/9	117 ±	−31.2	2.4
3 sem. après	19/9	107 ± 6.7		
4 sem. après	27/9	117.5 ± 10.8		

Ce retour à une pression normale accompagnant une régression concomittante dans l'hypertrophie des surrénales à la suite de la disparition de la cause responsable du stress, de même que l'absence de lésions rénales, sont autant d'observations qui nous portent à croire que l'hypertension obtenue au froid était due à l'hypersécrétion des hormones corticales.

(2) Hypertension due a un excès de NaCl dans l'eau de boisson

Protocole

Nous avons donné pendant 3½ mois du NaCl à 1% comme eau de boisson à 16 rats mâles d'un poids moyen de 300 grammes et nous avons observé que six d'entre eux manifestaient une élévation de pression moyenne de 65 mm. de Hg au bout de ce lapse de temps.

Après avoir vérifié, pendant quelques jours, que cette haute pression se maintenait définitivement à un niveau très élévé au-dessus de la normale, nous avons donné à ces animaux, par injection intrapéritonéale, 150 mg. d'acide ascorbique sous forme d'ascorbate de Na.

Immédiatement avant l'injection, 1 heure et 19 heures après, nous avons mesuré leur pression.

Résultats

Voici résumés dans la Fig. 3 et le Tableau II les résultats de cette expérience. On y voit que l'acide ascorbique abaisse sensiblement cette hypertension et que 19 heures après l'injection son effet hypotenseur se fait encore sentir.

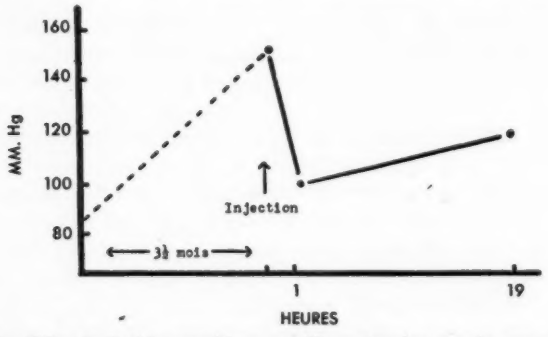

FIG. 3. Effet de l'acide ascorbique sur l'hypertension causée par un excès de NaCl dans l'eau de boisson.

Discussion

Selye et ses collaborateurs (15) ont trouvé que le stress produit de l'hypertension chez des rats recevant une solution isotonique de NaCl pour boisson, et ces auteurs, pour expliquer leurs résultats, ont directement impliqué les surrénales.

Sapirstein (13), après avoir reproduit cette hypertension en ne donnant qu'une solution hypertonique de chlorure de sodium à boire à ses rats, a montré que la surrénalectomie n'avait aucune influence sur cette élévation de pression,

TABLEAU II

INFLUENCE DE L'ACIDE ASCORBIQUE SUR L'HYPERTENSION CAUSÉE PAR DU NaCl À 1% PRIS COMME BOISSON

N°.	Poids initial gr.	Poids après 3½ mois gr.	Pression initiale, mm. Hg	Pression après 3½ mois, mm. Hg	Injection de 150 mg. Ac. asc., cc.	Pression 1 heure après, mm. Hg	Pression 19 heures après, mm. Hg
1	325	408	72	132	0.6	102	132
2	321	398	66	160	0.6	108	150
3	287	413	98	130	0.6	66	86
4	264	395	98	140	0.6	80	90
5	275	422	82	150	0.6	132	134
6	299	428	76	172	0.6	90	120
Moy.	295	411	82	147		96	119

pas plus d'ailleurs que la néphrectomie totale. Il a alors émis l'hypothèse que l'hypertension est due au déséquilibre qui apparaît dans la distribution des liquides; d'après lui les surrénales ou les reins n'auraient aucun rôle à jouer dans ce phénomène.

Quelle que soit l'étiologie de cette hypertension, il est intéressant de constater par notre expérience que l'acide ascorbique abaisse sensiblement cette élévation de pression.

(3) Hypertension due à une compression renale

Protocole

Nous avons provoqué cette hypertension en nous servant de la méthode décrite par Grollman (8). Cette méthode consiste à comprimer un rein au cours d'une première opération; cette compression se fait de la façon suivante: après avoir dégagé le rein de sa capsule, on l'entoure d'un fil de coton n° 10 en faisant une figure de huit et on serre suffisamment pour comprimer le rein sans que le fil pénètre dans le tissu rénal; au cours d'une deuxième opération, effectuée 12 jours après la première, on enlève l'autre rein. Les opérations ont été faites sans asepsie si ce n'est qu'avant de coudre la plaie nous y avons saupoudré du sulfathiazole.

Nous avons employé des rats mâles d'un poids moyen de 200 grammes. Le reste du protocole est identique à celui des deux expériences précédentes. Dès qu'un animal manifestait de l'hypertension soutenue pendant quelques jours, nous lui injections par voie intrapéritonéale 50 mg. d'acide ascorbique sous forme d'ascorbate de Na, et une heure après nous mesurions de nouveau sa pression.

Nous avons ensuite sacrifié tous les animaux et prélevé les organes qui, à l'examen macroscopique, paraissaient anormaux; nous les avons fixés au Bouin et colorés à l'hémotoxyline–éosine–bleu méthylène et au Mallory.

TABLEAU III

INFLUENCE DE L'ACIDE ASCORBIQUE SUR LA PRESSION DES RATS RENDUS HYPERTENSIFS PAR COMPRESSION RÉNALE UNILATÉRALE

N°	Pression			17/11	Pression					18/11	Pression	
	3/11	15/11	17/11		1 heure après	2 heures après	4 heures après	8 heures après	24 heures après		1 heure après	24 heures après
2	126	160	164	Inj. intra-péritonéale de 50 mg. Ac. asc.	92	136	142	166	170	Inj. intra-péritonéale de 0.5 cc. de $NaHCO_3$	170	174
264	110	178	150		96	90	110	134	150		152	164
262	124	160	168		138	124	122	148	170		172	164
Moy.	120	166	160		108	117	124	149	163		164	167

N.B. *Les rats ont été opérés le 3/11.*

Resultats

Examens microscopiques

Chez huit de ces rats hypertendus opérés cinq ou six mois auparavant, nous avons observé les mêmes lésions rénales et vasculaires déjà décrites par Selye (16) et Masson (10).

Ces lésions consistaient surtout en une dilatation modérée des tubules, avec un épithélium aplati, des cellules atrophiées et des magmats albumineux, des glomérules élargis où l'on observe un début d'hyalinisation de l'artériole afférente s'étendant parfois jusqu'entre les anses vasculaires. Chez quelques-uns nous avons observé une panartérite au niveau du mésentère.*

Effets de l'acide ascorbique

On peut voir, par le Tableau III, que dès le douzième jour après la première opération (compression d'un rein) les rats 2, 264 et 262 étaient déjà hypertendus. L'injection intrapéritonéale de 50 mg. d'acide ascorbique, sous forme d'ascorbate de sodium, eut pour effet de provoquer au bout d'une heure une chute de pression très marquée chez ces animaux. Mais dès huit heures après l'injection, ceux-ci étaient de nouveau hypertendus (Fig. 4). Le lendemain, l'injection de 0.5 cc. de $NaHCO_3$ n'eut aucun effet sur la pression de ces mêmes rats.

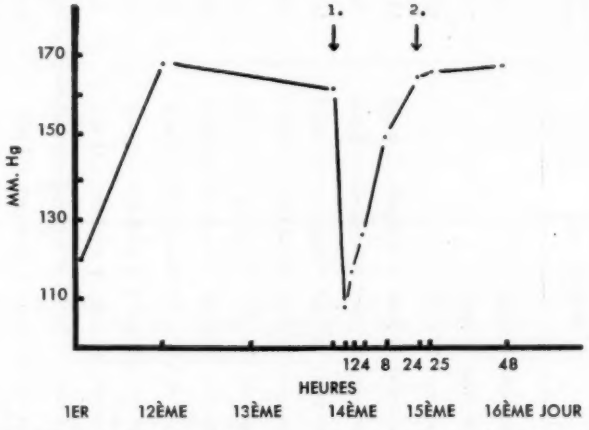

FIG. 4. Effet de l'acide ascorbique sur la pression des rats rendus hypertendus par compression rénale unilatérale.
1. Injection: acide ascorbique. 2. Injection: $NaHCO_3$.

Comme on peut le voir au Tableau IV, l'acide ascorbique eut le même effet sur les rats: 257, 254, 249, 200 et 258, chez qui l'hypertension n'est apparue qu'après néphrectomie du rein droit. Quelques uns reçurent du NaCl à 1% comme eau de boisson, afin d'accélérer l'apparition de l'hypertension.

** Ces lésions ont été identifiées grâce au généreux concours du Dr. Carlton Auger, directeur du Département d'Anatomo-Pathologie de la Faculté de Médecine de l'Université Laval.*

TABLEAU IV

EFFET DE L'ACIDE ASCORBIQUE SUR L'HYPERTENSION RÉNALE EXPÉRIMENTALE

Numéro	Traitements	Pression initiale	Pression après traitement	Injection	1 heure après	Différence	Lésions
2	Compression d'un rein: 12 jours	126	164	Asc. Na	92	−72	
264	Compression d'un rein: 12 jours	110	150	"	96	−54	
262	Compression d'un rein: 12 jours	124	168	"	138	−30	
257	Compression d'un rein: 12 jours + nephrect. (1 mois)	114	196	"	128	−68	
254	Compression d'un rein: 12 jours + nephrect. (1 mois)	98	156	"	136	−20	
9	Compression d'un rein: 12 jours + nephrect. (1 mois)	122	160	$NaHCO_3$	164	+ 4	
267	Compression d'un rein: 12 jours + nephrect. (1 mois)	118	156	"	150	− 6	
249	Compression d'un rein: 12 jours + nephrect. (1 mois) + NaCl (15 jr.)	82	168	Asc. Na	130	−38	++
200	Compression d'un rein: 12 jours + nephrect. (1 mois) + NaCl (15 jr.)	104	142	"	106	−36	−
258	Compression d'un rein: 12 jours + nephrect. (3½ m.) + NaCl (2½ m.)	88	182	"	136	−46	−
266	Compression d'un rein: 12 jours + nephrect. (3½ m.) + NaCl (2½ m.)	80	130	"	130	0	+++
245	Compression d'un rein: 12 jours + nephrect. (3½ m.) + NaCl (3 mois)	92	146	"	138	− 8	+
254	Compression d'un rein: 12 jours) + nephrect. (6 mois)	98	162	"	156	− 6	++++
200	Compression d'un rein: 12 jours + nephrect. (3½ m.) + NaCl (3 mois)	104	160	"	162	+ 2	++++
2	Compression d'un rein: 12 jours + nephrect. (6 mois)	126	140	"	150	+10	+

L'injection de $NaHCO_3$ aux nº 9 et 267 n'eut aucun effet sur leur pression.

Enfin, 6½ à 7 mois après le début de l'expérience, l'injection de 50 mg. d'acide ascorbique aux cinq derniers rats traités de la façon indiquée dans le Tableau IV, n'eut aucun effet sur leur pression. On remarquera que les trois derniers sont les mêmes que les nºs 2, 254, 200 du début chez qui l'acide ascorbique avait diminué la pression alors qu'ils n'avaient été opérés que depuis peu.

Nous avons constaté, chez ces cinq derniers animaux, des lésions rénales assez avancées. Les signes + dans le Tableau IV rendent compte d'une façon arbitraire et grossière de la sévérité des lésions chez chaque animal étudié.

L'effet de l'acide ascorbique sur la pression des différents groupes de rats est résumé dans la Fig. 5.

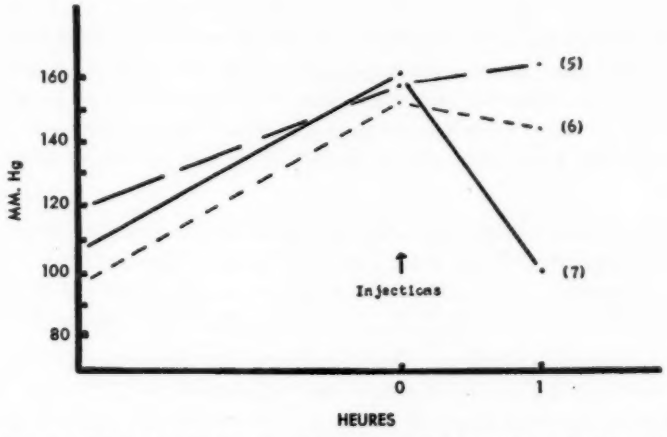

FIG. 5. Effet de l'acide ascorbique sur l'hypertension rénale expérimentale. ——— = Injection d'acide ascorbique: rats opérés depuis un ou deux mois. — — — = Injection de 0.5 cc. $NaHCO_3$. — — — — — = Injection de 50 mg. d'acide ascorbique: rats opérés depuis six mois.

Discussion

Par la première expérience, nous avons confirmé l'action hypotensive de l'acide ascorbique chez l'animal rendu hypertendu par une longue exposition au froid. La disparition de l'hypertension chez ces animaux quand ils sont ramenés à la température normale, l'absence de lésions rénales et la régression de l'hypertrophie des surrénales nous indiquent que nous avons fort probablement affaire ici à une hypertension d'origine hormonale.

L'acide ascorbique abaisse aussi l'hypertension provoquée par un excès de NaCl dans l'eau de boisson.

Finalement, l'acide ascorbique a le même effet hypotenseur sur l'hypertension rénale expérimentale, excepté quand les lésions rénales et vasculaires sont trop avancées.

Bien que tous ces résultats aient été obtenus dans chaque cas, sur un nombre plutôt restreint d'animaux, nous croyons pouvoir affirmer de façon générale que l'acide ascorbique a un effet hypotenseur marqué chez les animaux souffrant d'hypertension expérimentale.

On reconnaît suffisamment aujourd'hui le rôle primordial joué par les surrénales dans ces différentes hypertensions, si ce n'est le cas encore incertain de l'hypertension produite par excès de NaCl dans l'alimentation, pour pouvoir leur attribuer la même étiologie hormonale et considérer l'action hypotensive de l'acide ascorbique comme relevant du même mécanisme dans chaque cas.

Il est intéressant de noter que dans l'hypertension rénale, chez les animaux devenus hypertendus plusieurs mois après leurs opérations et chez lesquels peuvent être observées des lésions rénales et vasculaires assez avancées, l'acide ascorbique n'a plus aucun effet hypotenseur. Il est donc fort possible, croyons-nous, que Wakerlin (19) n'ait observé aucune baisse de pression à la suite de l'injection d'acide ascorbique à son chien souffrant d'hypertension rénale, justement parce que celui-ci devait avoir des lésions rénales trop avancées.

Nos résultats, comme celui de Wakerlin, peuvent même, croyons-nous, être considérés comme une indication que dans la phase chronique de l'hypertension rénale expérimentale, l'élévation de la pression est due beaucoup plus aux lésions rénales et vasculaires qu'à n'importe quel mécanisme humoral possible.

Plusieurs chercheurs d'ailleurs, dont Goldblatt (7), Haynes et Dexter (9), Masson (10), sont d'avis que le mécanisme responsable de l'élévation de la pression dans les derniers stages de l'hypertension rénale expérimentale diffère du mécanisme initial.

Résumé

(1) L'acide ascorbique abaisse l'hypertension provoquée expérimentalement par les trois moyens suivants: (1) exposition à un stress continu (longue exposition au froid), (2) absorption d'une solution isotonique de NaCl, (3) compression rénale et néphrectomie unilatérale.

(2) L'hypertension et l'hypertrophie surrénalienne provoquée par une longue exposition au froid, disparaissent quand on ramène les animaux à la température normale.

(3) Dans sa phase initiale, l'hypertension rénale expérimentale est abaissée par l'acide ascorbique mais au cours de sa phase chronique, elle résiste à l'effet hypotenseur de cette substance.

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PLASMA ASCORBIC ACID AND PITUITARY–ADRENAL ACTIVITY[1]

By F. R. Skelton and C. Fortier

Abstract

In a study of the relationship of ascorbic acid metabolism to pituitary-adrenal function in the rat, the effect of stress, adrenalectomy, hypophysectomy, and administration of an adrenal cortical extract on the plasma ascorbic acid concentration was determined. Surgical trauma and cold elicited a triphasic, and scalding a biphasic, response in both intact and adrenalectomized animals. The plasma ascorbic acid concentration decreased after adrenalectomy and hypophysectomy but returned to normal or above normal level following the administration of an adrenal cortical extract. It is suggested that the pituitary–adrenal system plays a facilitating role in the increased synthesis of ascorbic acid elicited by stress.

Introduction

The adrenal ascorbic acid changes resulting from pituitary–adrenal activation are well known (8, 16), while the influence possibly exerted by this system on ascorbic acid metabolism remains obscure. Some relationship is suggested by the markedly increased urinary excretion of the vitamin observed in the rat during chronic stress (19) and after treatment with a wide variety of substances (1). However, Ralli (11) considers urinary excretion a poor criterion of ascorbic acid metabolism in this species since many factors, including glomerular filtration rate, tubular reabsorption, plasma level, and rate of vitamin synthesis, influence the amounts lost in the urine. The plasma level was accordingly chosen as a more suitable index, and its variations studied following exposure to stress, adrenalectomy, hypophysectomy, and administration of an adrenal cortical extract.

Adult, male piebald rats weighing 150–200 gm. were used and fasted in all experiments, for a uniform period of 24 hr. prior to killing. Groups of six to eight animals were killed at each time interval throughout the series. The blood, collected from the carotid artery and prevented from clotting by calcium oxalate, was immediately centrifuged and the plasma analyzed for its ascorbic acid content by the method of Roe and Kuether (14).

Experiments and Results

A. *Effect of Stress on Plasma Ascorbic Acid in the Intact Rat*

Three types of alarming agents were studied for their effects on plasma ascorbic acid. In one experiment surgical trauma was produced by crushing a loop of intestine with a hemostat (six times each of 10 sec. duration) while the animals were under sodium pentobarbital anesthesia. As barbiturates reportedly alter ascorbic acid metabolism (3), this factor was controlled by including an anesthetized group. Thus the difference between the values

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obtained in these two groups represents the true effect of the surgical trauma. Exposure to cold (+ 2° C. to 4° C.) and to scalding (partial immersion in water at 70° C. for 15 sec.) were the stresses used in the second and third experiments respectively. The results are shown in Table I, while the percentage deviation from normal of the values obtained with cold are plotted against time in Fig. 1.

TABLE I*

Treatment	Time intervals						
	0**	30 Min.	1 Hr.	3 Hr.	6 Hr.	12 Hr.	24 Hr.
I. Effect of stress on the plasma content of ascorbic acid							
Trauma	1306 ± 75	816	1049	1443	1073	992	687
Cold	743 ± 70	573 ± 77	590 ± 83	660 ± 80	847 ± 72	947 ± 228	880 ± 46
Scalding	1200 ± 180		1147 ± 129	1257 ± 175	1537 ± 149	1355 ± 204	983 ± 132
II. Effect of adrenalectomy and stress in the adrenalectomized animal on the plasma content of ascorbic acid							
Adrenalectomy	743 ± 70			637 ± 45		550 ± 95	523 ± 67
Adrenalectomy + cold	833 ± 61	667 ± 66	750 ± 85	1150 ± 115	1283 ± 98	770 ± 78	920 ± 83
Adrenalectomy + scalding	586 ± 175		843 ± 110	807 ± 87	1076 ± 165	840 ± 129	1175 ± 146
III. Effect of adrenal cortex extract on the plasma content of ascorbic acid							
Adrenalectomy + cortical extract	1466 ± 98	1627 ± 109	2073 ± 92	2057 ± 133	2140 ± 132	1937 ± 85	
IV. Effect of hypophysectomy on the plasma content of ascorbic acid							
Hypophysectomy	715 ± 66			620 ± 91		583 ± 113	410 ± 165

* *The values represent the means of six to eight determinations, followed by the standard errors.*

** *Zero time represents the basic control value for each experimental series. The wide range of variation under basal conditions is attributed to the many factors conditioning the synthesis of the vitamin in the rat.*

A triphasic response pattern was observed after exposure to cold and surgical trauma, which was characterized by a fall at 30 min., followed by a transient rise above the normal level with a progressive decline thereafter. The response to scalding was biphasic since no initial fall occurred at one hour, the earliest period studied in this experiment, or at 15 and 30 min. after scalding in additional experiments.

B. Effect on Plasma Ascorbic Acid of Adrenalectomy and of Stress in the Adrenalectomized Rat

The plasma content of ascorbic acid was determined at several intervals up to 24 hr. following bilateral adrenalectomy. In addition, the effect of stress on this metabolite in the adrenalectomized rat was studied using cold and scalding

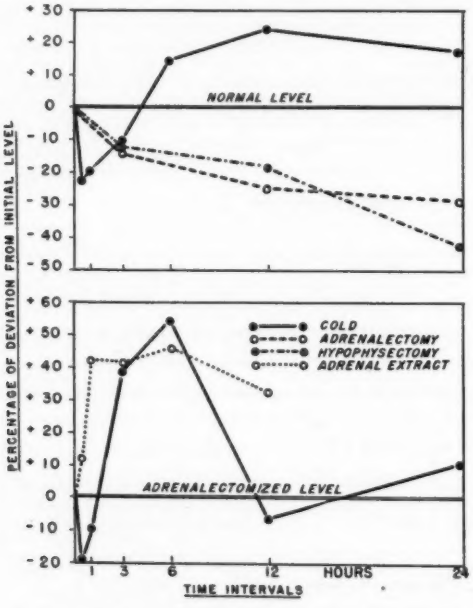

FIG. 1. The upper half of the figure illustrates the percentage changes in the plasma ascorbic acid concentration of the rat following exposure to cold, adrenalectomy, and hypophysectomy. The lower half similarly shows the effects of cold and treatment with adrenal cortical extract (Upjohn's Lipo-Adrenal Cortex) on the plasma content of this vitamin in the adrenalectomized rat.

as representative alarming stimuli. Each group was killed 48 hr. after adrenalectomy. Following adrenalectomy a slow, progressive fall in the plasma ascorbic acid content occurred, reaching a level 30% below normal after 24 hr. (Table I, Fig. 1). Furthermore cold and scalding in the adrenalectomized rats reproduced the same pattern of plasma ascorbic acid response as in the intact animal.

C. Effect of Adrenal Cortical Extract on Plasma Ascorbic Acid in Adrenalectomized Rats

The significant decrease of plasma ascorbic acid following adrenalectomy led us to study the effects of cortical extract in the adrenalectomized animal.

Lipo-adrenal cortex extract (Upjohn) in a dose of 0.4 ml. per rat was administered subcutaneously 24 hr. after adrenalectomy. A rapid and significant rise in the level of plasma ascorbic acid of at least 12 hr. duration was observed following this treatment (Table I, Fig. 1).

D. Effect of Hypophysectomy on Plasma Ascorbic Acid

Plasma ascorbic acid levels were determined at intervals up to 24 hr. postoperatively in the hypophysectomized rat. A progressive fall occurred, similar to that observed after adrenalectomy (Table I, Fig. 1). Further data indicate that stress in the hypophysectomized rat also reproduces the same pattern of plasma ascorbic acid response as in the normal animal.

Discussion

The control of ascorbic acid synthesis remains poorly understood despite the many conditions known to affect it. Thus vitamin A deficiency (10), insulin (4, 12), and thyroxine (2, 6) administration decrease its concentration in the plasma while such varied substances as chloretone (15), paraldehyde (3), barbiturates (3), salicylates (13), sulphonamides (5), and terpenes (9), increase both the plasma concentration and urinary excretion. This very multiplicity suggests some common mechanism of action on ascorbic acid metabolism which may well be the stress factor acting either independently or through the pituitary–adrenal system.

Among other observations suggesting a relationship between the endocrine system and ascorbic acid synthesis and metabolism in the rat is its increased excretion during chronic exposure to cold (19), and the decreased urine and tissue content following hypophysectomy and adrenalectomy (18, 20). A significant but transient (24 hr. duration) rise in plasma level with a concomitant increase in the liver concentration of ascorbic acid following nonfatal hemorrhage in the intact rat has been reported by Sayers *et al.* (17). Previous hypophysectomy allegedly prevents the plasma response but fails to alter the increase in liver content of the vitamin. A sixfold increase in plasma ascorbic acid accompanied by a progressive fall in the liver concentration was observed in the terminal stages following fatal hemorrhage. No initial decline in plasma level, such as we have observed following trauma or exposure to cold, was found in either instance.

In contrast Sutton (18) observed that neither hypophysectomy nor adrenalectomy prevented the increase in ascorbic acid excretion normally produced by chloretone while our results demonstrate that a given stress reproduces essentially the same plasma ascorbic acid response pattern in both the intact and adrenalectomized rat. However, the importance of the pituitary–adrenal system in the maintenance of ascorbic acid synthesis is emphasized by the marked decline in plasma levels following adrenalectomy and hypophysectomy and the striking elevation induced by lipo-adrenal cortex extract.

In species unable to synthesize vitamin C, the urinary excretion and tissue content are decreased during stress (19), undoubtedly resulting from increased tissue utilization, since many studies have shown that increased requirements are caused by increased general metabolism (2, 6, 7). However, in species which synthesize ascorbic acid, stresses such as cold which cause increased metabolism, probably induce greater utilization of the vitamin than can be met through synthesis, thus resulting in a decreased initial plasma level. As synthesis of ascorbic acid is soon in excess of the tissue requirements, a transient rise of its concentration in the plasma occurs. The failure of other stresses to cause an initial decrease in plasma level suggests a closer correspondence of synthesis to tissue requirements under these conditions.

Summary

1. Plasma ascorbic acid levels have been studied in relation to pituitary–adrenal function in the rat.

2. Surgical trauma and cold produced a triphasic, and scalding a biphasic, response in both normal and adrenalectomized animals.

3. The level of plasma ascorbic acid progressively decreased after adrenalectomy and hypophysectomy but returned to normal or above normal levels following the administration of adrenal cortical extract.

4. The pituitary–adrenal system is important in the maintenance of normal ascorbic acid metabolism in the intact animal. The increased synthesis observed during stress is facilitated by, though not mediated through this system. It is suggested that the initial fall in plasma level following certain types of stress is the result of a temporary imbalance between tissue utilization and synthesis.

Acknowledgments

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SOME THEORETICAL ASPECTS OF RADIOAUTOGRAPHY[1]

By N. J. Nadler

Abstract

The purpose of radioautography (also called autoradiography or autography) is to identify the presence of radio-elements within microscopic structures by the action of the emitted radiations on photographic emulsions. Since the quality of the photographic image depends on its resolution and density, a theoretical study of these two characteristics was made. For resolution studies, a theory based on an inverse square law relationship for β-ray intensity is expanded to include various geometric shapes of radioactive sources encountered in microscopical preparations. The formulae provide a means to calculate the resultant distribution of photographic blackening (density), from which it is possible to determine how the resolving power is influenced by the geometrical relations between the tissue preparation and photographic emulsion. For density studies, a theory is presented whereby it has been possible to arrive at the average number of impacts of given β-particles required to render a medium contrast emulsion grain developable. The basis of the theory rests on calculating the mean energy dissipated by a β-particle in traversing a silver bromide grain, and determining from this the number of reduced silver atoms deposited per grain. Realizing the average number of silver atoms necessary to render a grain developable, the required number of impacts by a β-particle may then be calculated. An example is given in which β-particles of a mean energy equal to that given off by radioiodine are made to react with the so-called medium contrast emulsion.

Radioautography is a technique for detecting the location of radio-elements in tissues. The method consists of applying a photographic emulsion to a tissue section, allowing sufficient time for the emitted radiation to act on the emulsion, and developing and fixing the preparation as in ordinary photography. The sites of radioactivity in the tissue section can then be identified by the accumulation of microscopic black silver granules (the radioautographic image) adjacent to those areas in the section which contain the radioactive element.

An extensive review of the theory and practice of radioautography and its application to biology and medicine has been presented in another paper (5). It is the purpose of this report to amplify the mathematical and physical details of that theory.

The density and resolution of the radioautographic image are the two main theoretical considerations concerned. The relevant factors involved are:

(*a*) the geometrical relationship of the radioactive source and overlying emulsion;

(*b*) the energy and exposure of the radiation;

(*c*) the characteristics of the emulsion.

The following discussion will deal with some of the information which is available for the study of the geometrical and energy factors in the radioautography of β-ray emitting isotopes.

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The Geometrical Relations of the Source and Emulsion

The autographic preparation consists of the section, the overlying emulsion, and the space between the two, i.e., the interspace (Fig. 1). To analyze the

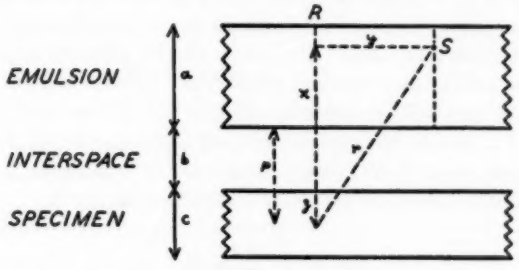

FIG. 1. Schematic representation of the action of a radioactive point source, P, on a photographic emulsion.
P is a point of radioactivity in the specimen.
S is any point in the emulsion.
a = thickness of the emulsion.
b = interspace between specimen and emulsion.
c = thickness of specimen.
PR is a perpendicular through P, assuming that edges of specimen and emulsion are parallel to one another.
x, y, z—as indicated by dotted lines.
r = distance PS.
p = $z + b$, i.e., distance of point source P from edge of the emulsion. (Adapted from Pelc (7).)

effects of geometrical variations of these components, Pelc (7) considered a source of radioactivity confined to a point in the tissue section (point source) and computed the total amount of radiation received throughout the thickness of the emulsion at any distance from the point source. This computation was readily extended to the case where the radioactivity was confined to a line perpendicular to the section (line source (7)).

Postulating a direct variation between photographic density of blackening and β-radiation exposure (i.e., sum of incident β-particles), which is probably valid in the low range of exposures (1), the resultant distribution of density, and, therefore, resolution can be inferred from these results.

The following is also implied in Pelc's analysis:

(*a*) β-radiation decreases in intensity (i.e., concentration of β-particles) according to the inverse square law, as if β-particles were travelling in straight lines;

(*b*) the radioactive intensity of the source is uniform;

(*c*) no account is taken for the fact that the β-particles are continuously losing energy, thereby affecting the photographic emulsion differently along their course.

Although these assumptions are only approximations, the theoretical data based on them do correlate with recent experimental density measurements.

Furthermore, they are of help in indicating the best conditions for radioautography (5) and the necessity of taking into account the geometrical component in attempting quantitative estimations of radioautographic images.

The remainder of this section deals with Pelc's initial contribution and the extension of his theory to cases when the source of radioactivity has the shape of a plane or cylindrical solid. The latter can be adapted to the problem of estimating the radioiodine content of thyroid follicles where the colloid in microscopic section assumes this solid circular cylindrical shape.

The effect of a *point source of radioactivity* is illustrated in Fig. 1, in which the photographic density (D) in the emulsion, at a distance y from the perpendicular through the point source (P) of radioactivity is:

$$^{P}D = \int_{x=0}^{x=a} \frac{A\,^{P}Q}{r^2}\,dx$$

where, ^{P}D refers to the density for a point source of radioactivity;

A is the sensitivity constant of the emulsion (dependent on the qualities of the photographic emulsion and on the energy of the incident β-rays); and,

^{P}Q is the total radiation resulting from the source at P, i.e., the total number of emitted β-particles.

If p, the distance of the point source (P) from the edge of the emulsion, and y, indicated by the broken line (Fig. 1), are kept constant, then the density due to the point source is equal to:

$$A\,^{P}Q \int_{x=0}^{x=a} \frac{dx}{(p + x)^2 + y^2}.$$

It follows, mathematically, that,

when $y > 0$,

$$^{P}D = A\,^{P}Q \frac{1}{y} \tan^{-1} \frac{ay}{y^2 + p(p + a)} = A\,^{P}Q\,^{P}D' \qquad \text{(Equation I}a\text{)}$$

where, $^{P}D'$ has been substituted for

$$\frac{1}{y} \tan^{-1} \frac{ay}{y^2 + p(p + a)}$$

and when $y = 0$,

$$^{P}D_{MAX} = A\,^{P}Q \frac{a}{p(p + a)} = A\,^{P}Q\,^{P}D'_{MAX} \qquad \text{(Equation I}b\text{)}$$

where, $^{P}D'_{MAX}$ has been substituted for

$$\frac{a}{p(p + a)}.$$

Calculations based on Equations Ia and Ib for several variations in p and y give results shown graphically in Fig. 2.

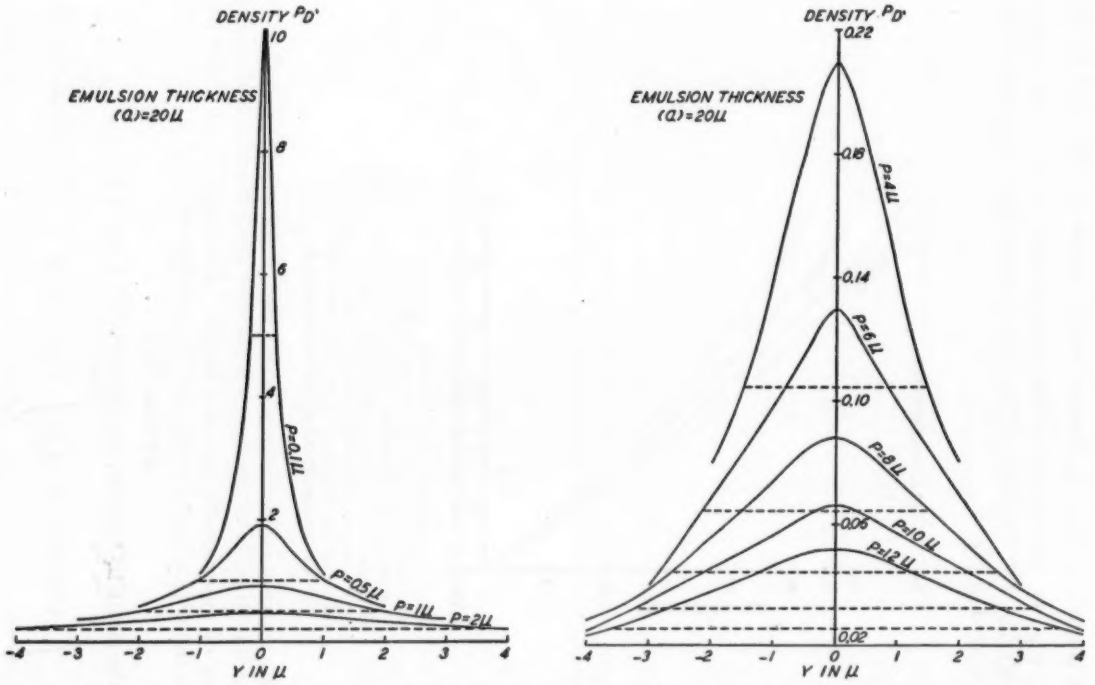

FIG. 2. Influence of interspace on resolution and photographic density ($^PD'$) of the autographic image resulting from a point source of radioactivity. A series of characteristic curves showing the density in arbitrary units at various distances y in the emulsion from the perpendicular through point source (see Fig. 1), for various point sources at distances, p, from the edge of the emulsion. The dotted lines measure the resolution.

These curves illustrate how the increase in the distance p decreases the maximum density and resolution obtainable with a point source, when the emulsion thickness is 20 μ.

At Pelc's suggestion, the resolution was defined as the dispersion or width of the characteristic curve (Fig. 2) at the level of ${}^{P}D'$ where it is equal to $\frac{{}^{P}D'_{MAX}}{2}$. It is obvious that the smaller is this resolution (indicated by the broken lines, Fig. 2), the sharper is the photographic image. The relation between resolution and distance of the point source from the edge of the emulsion is plotted in Fig. 3.

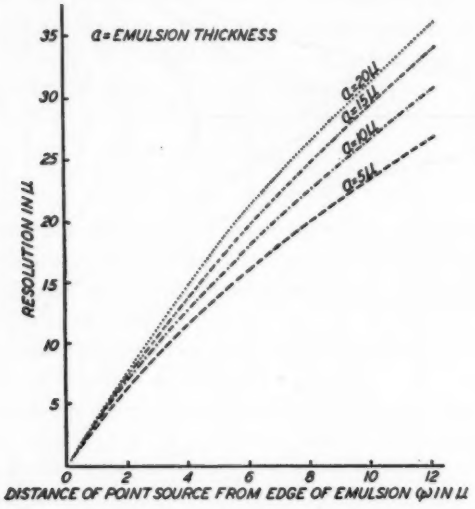

FIG. 3. Influence of emulsion thickness on the resolution of a point source autograph. Curves illustrating the relation between resolution and distance, p, of a point source from edge of emulsion for various emulsion thicknesses. It is concluded that the emulsion thickness is of relatively minor significance compared with the distances p.

Using a *line source of radioactivity* perpendicular to the section (Fig. 1), it is assumed that the intensity of the source is uniform along the line, and results in an amount of radiation ${}^{L}Q$ per unit length. The intensity at P for the element Δz is then ${}^{L}Q\Delta z$, and the density, ${}^{P}D$, as before, is equal to:

$$A({}^{L}Q\Delta z){}^{P}D',$$

so that for the entire line source, the density, ${}^{L}D$, is equal to

$$\int_{z=0}^{z=c} A\,{}^{L}Q\,{}^{P}D'dz = A\,{}^{L}Q\int_{p=b}^{p=b+c} {}^{P}D'dp = A\,{}^{L}Q\,{}^{L}D' \qquad \text{(Equation II)}$$

where, ${}^{L}D'$ has been substituted for

$$\int_{p=b}^{p=b+c} {}^{P}D'dp.$$

In Table I, Equation II has been applied to show the influence of the emulsion thickness, section thickness, and interspace on the resolution and maximum density (i.e., density over line source itself) obtainable with a line source of radioactivity.

TABLE I

THE RESOLUTION AND DENSITIES RESULTING FROM A LINEAR SOURCE OF RADIOACTIVITY UNDER VARIOUS CONDITIONS OF SECTION, EMULSION, AND INTERSPACE THICKNESS

Emulsion thickness (μ)	Interspace (μ)	Section thickness (μ)	Resolution (μ)	Max. density
5	0.1	5	2.3	3.2
5	0.1	10	2.8	3.5
10	0.1	5	2.7	3.5
10	0.1	10	3.7	3.9
15	0.1	5	3.3	3.7
15	0.1	10	4.4	4.1
5	0.5	5	5.7	1.8

Similarly, in Fig. 4, using a *plane source of radioactivity* perpendicular to the section and assuming a uniform amount of emitted radiation, ${}^{Pl}Q$, per

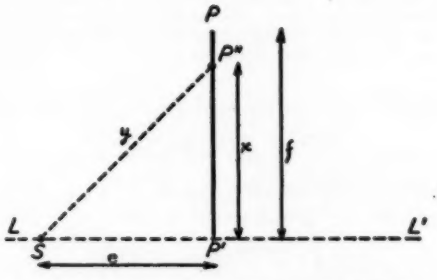

FIG. 4. Plan view of emulsion with PP' the projection on the emulsion of the radioactive *plane source* of length f, present in the histological section. LL' is any line in the emulsion perpendicular to the projection of the source. S is any point on LL'.

unit plane area, the density at S, distance e from the plane, for any element Δx in the plane projection, is:

$$ {}^{L}D = A({}^{Pl}Q\Delta x){}^{L}D'. $$

Therefore, for the entire plane of length f, the photographic density, ${}^{Pl}D$, is:

$$ A{}^{Pl}Q\int_{x=0}^{x=f} {}^{L}D'dx = A{}^{Pl}Q{}^{Pl}D' \qquad \text{(Equation III)} $$

where, ${}^{PL}D'$ has been substituted for

$$\int_{x=0}^{x=f} {}^{L}D'dx.$$

For any given thickness of emulsion section, tissue section, and interspace, the value of ${}^{PL}D'$, at any distance e, can be obtained by graphically integrating the curve ${}^{L}D'$ vs. x over the appropriate interval of x. The curve ${}^{L}D'$ vs. x is simply obtained from the results of Equation II (${}^{L}D'$ vs. y), and from the relation $y = \sqrt{x^2 + e^2}$.

Again, in Fig. 5, for a *solid circular cylindrical source*, assuming a uniform amount of emitted radiation, ${}^{C}Q$, per unit volume, the photographic density

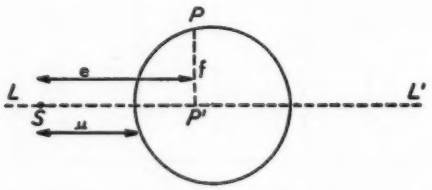

FIG. 5. Plan view of emulsion with a circle being the projection on the emulsion of the radioactive *solid cylinder source* of radius R, present in the histological section. LL′ is a line in the emulsion perpendicular to the projection of the source. S is any point on LL'.

at S for a plane length, f, in an element Δe of the solid circular cylindrical projection, is:

$${}^{PL}D = A({}^{C}Q\Delta e){}^{PL}D'$$

and, for the entire source, the density, ${}^{C}D$, is:

$$A{}^{C}Q\int_{e=u}^{e=u+2R} 2{}^{PL}D'de = A{}^{C}Q{}^{C}D'$$

where, ${}^{C}D'$ has been substituted for

$$\int_{e=u}^{e=u+2R} 2{}^{PL}D'de.$$

${}^{C}D'$ is obtained by graphical integration of the appropriately constructed curve, ${}^{PL}D'$ vs. e, where ${}^{PL}D'$ was obtained from Equation III, the limits of integration being determined by the relation,

$$f = \sqrt{R^2 - (u + R - e)^2}.$$

The results for the distribution of photographic density for a cylinder of radius 20 μ are shown in Table II and Fig. 6. Resolution was arbitrarily

TABLE II

THE RESOLUTION OBTAINED WITH CYLINDRICAL SOURCES OF RADIOACTIVITY UNDER VARIOUS CONDITIONS OF SECTION, EMULSION, AND INTERSPACE THICKNESS

Emulsion thickness (μ)	Interspace (μ)	Section thickness (μ)	Resolution (μ)	Curve No. (Fig. 6)
5	0.1	2	5.0	I
5	0.1	5	6.0	III
5	0.5	5	6.4	II
10	0.1	5	8.0	IV
20	0.1	5	9.0	V
35	0.1	5	10.2	VI

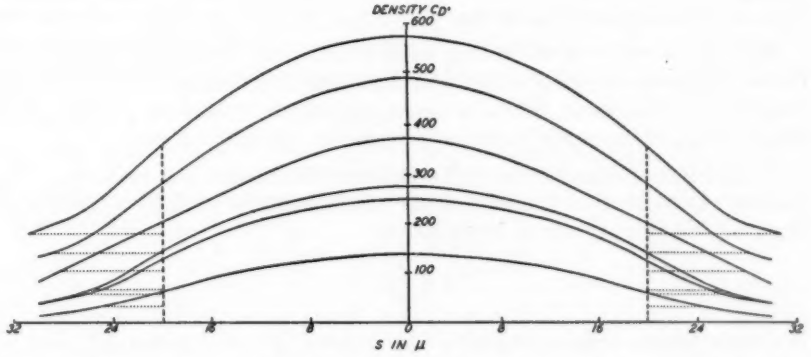

FIG. 6. Geometric factors influencing the autograph of a cylindrical source. Curves illustrating the blackening density and distribution, $^{C}D'$, in arbitrary units, at distances, S, from the projected center of a solid circular cylindrical source of 40 μ diameter. The dotted lines measure the resolution. The Roman numerals refer to the conditions given in Table II.

redefined as the distance from the edge of the source to a point on the characteristic curve (Fig. 6) where the density was one-half of that over the edge of the source (as indicated by the broken line).

A somewhat modified approach to this problem of geometry, which has recently been applied to the subject of quantitative autography by the author, is to determine the radiation intensity at different emulsion levels on the basis of an inverse square relationship. The only difference is that the initial integration over the depth of the emulsion is omitted, so that, referring to Fig. 1,

$$^{P}D = A^{P}Q \frac{1}{(p + x)^2 + y)^2}$$

for a given emulsion level, x. The remaining manipulations are as before. Finally, the mean density per unit area at any emulsion level for a given source of radioactivity may be determined. Preliminary experimental results

obtained by grain counting at different emulsion levels seem to substantiate the theoretical curves of density distribution.

The Energy of the Radiation

The mechanism of the photographic action of ionizing particles has been reviewed by Demers (3). The photographic emulsion contains "grains" which consist mainly of silver bromide crystals. Essentially, the radioactive particle releases energy to excite orbital electrons into the conduction band of the silver bromide molecule. These electrons are trapped at specific crystal irregularities and then attract and neutralize silver ions. The deposition of elemental silver, the so-called "photolytic silver", forms the latent image. If sufficient photolytic silver is deposited in the crystal, it becomes developable, that is, a developer can then chemically reduce ionic silver to metallic silver which precipitates about the development centers, making the crystals appear as black granules.

For a given emulsion, the photographic efficiency of the ionizing particle will depend on the space rate of energy loss of that particle through a silver bromide medium. Furthermore, the total range, and therefore the total photographic effectiveness, of the particle will be influenced by its initial energy.

A β-particle dissipates energy by means of ionization and radiation. In general, the ionization, I, produced per unit distance of path by a particle of charge E and velocity v, is of the order of:

$$I \propto \frac{E^2}{v^2}$$

If a more precise representation for the ionizing power of a charged particle within silver bromide be desired, it is possible to arrive at more definite conclusions regarding the interaction of a β-particle with a photographic emulsion. For this, recourse must be made to a formula arrived at by Bethe, Block, and others (2, 8):

$$-\left(\frac{dT}{dx}\right)_{\text{ioniz}} = \frac{2\pi e^4 NZ}{m_0 v^2}\left\{2\log\frac{2m_0v^2}{\bar{I}} - \log\left(1-\frac{v^2}{c^2}\right) - \frac{v^2}{c^2}\right\}^*$$

* (*a*) *This formula was derived on the basis of collisions of β-particles with orbital electrons. For its application, however, two conditions must be satisfied:*

(*1*) *The velocity of the β-particle, v, must be significantly greater than the velocity of the orbital electrons. This can be shown to be so if m_0v^2 be significantly greater than the excitation energy, I, of the orbital electrons. For a β-particle with kinetic energy 0.21 mev. (mean energy for I^{131} emitted β-rays), m_0v^2 is 0.26 mev. On the other hand, those electrons located in the innermost orbit (K shell) have the greatest excitation potentials, of which the values for silver and bromine are 25.5 kev. and 13.5 kev., respectively.*

(*2*) $\frac{Ze^2}{hv}$ *must be significantly less than unity. Substituting appropriate values, the expression is equal, in this case, to 0.078.*

It is clear, then, that both conditions are satisfied for media of Ag ($Z = 47$) and Br ($Z = 35$). It is interesting to note, however, that because of the relatively large mass of the α-particle, and hence

where $-\left(\frac{dT}{dx}\right)_{\text{ioniz}}$ = rate of loss of energy by ionization per unit length of path by a β-particle,

e = charge on the electron, 4.77×10^{-10} esu.,

N = number of atoms per cc. of medium through which the β-particle passes,

Z = average nuclear charge of atoms of that medium,

m_0 = rest mass of the electron, 8.99×10^{-28} gm.,

v = velocity of the β-particle in cm./sec.,

$\frac{v}{c}$ = relativistic expression, where c is the velocity of light,

$\bar{I}$ = average excitation potential of the atoms constituting the medium.

Account has also been taken for the energy lost by radiation due to acceleration of a charged particle in atomic fields (8). The final results for the total space rate of energy loss (ionization and radiation) are listed in Table III.

TABLE III

TOTAL SPACE RATE OF ENERGY LOSS BY IONIZATION AND RADIATION FOR β-PARTICLES OF VARIOUS KINETIC ENERGIES

Kinetic energy, T, mev.	$-\left(\frac{dT}{dx}\right)_{\text{ioniz}}$ mev./cm.	$-\left(\frac{dT}{dx}\right)_{\text{rad.}}$ mev./cm.	$-\left(\frac{dT}{dx}\right)_{\text{total}}$ mev./cm.
0.10	18.4	0.09	18.5
0.20	12.9	0.13	13.0
0.30	10.7	0.17	10.9
0.50	9.00	0.22	9.22
0.90	6.19	0.38	8.57
1.10	8.00	0.45	8.45
1.40	8.00	0.57	8.57
1.80	7.94	0.73	8.67

smaller velocity for the same energy, limitations are automatically imposed on the application of this formula to the dynamics of the α-particle.

(b) The calculation of $\bar{I}$ presents an unfortunate obstacle to a more accurate evaluation of $-\frac{dT}{dx}$. Rasetti (8) states that $\bar{I}$ can be considered equal to $13.6 \times Z$ in ev. Bethe (2), however, indicates clearly that $\bar{I}$ is not quite any such simple number. Using gold as a medium for β-particles, they found that for precise correlation with experiment $\bar{I}$ was 520 ev. while Z had to be taken as 61.4 because the K and L electrons were not affected. This implied that for the evaluation of $\bar{I}$ the coefficient of Z is less than 10, but here the electrons which have the highest excitation potential, namely, K, L, and M electrons, are not involved. However, because of the log term in the formula, the discrepancy involved in the choice of $\bar{I}$ does not significantly affect the result. A coefficient of 11 is thus tentatively accepted, so that for AgBr containing an average nuclear charge of 41, $\bar{I}$ is 451 ev.

(c) From the configuration of the silver bromide lattice (cubic with mean distance between any two atoms 2.88 Å), it follows that the concentration of both silver and bromine atoms is 4.19×10^{22} atoms per cc. of silver bromide crystal.

Fig. 7 shows the relation between the rate of total energy loss of a β-particle and the energy of that particle in a medium of silver bromide. It is then evident by comparing the mean energies of beta-rays emitted from C^{14}

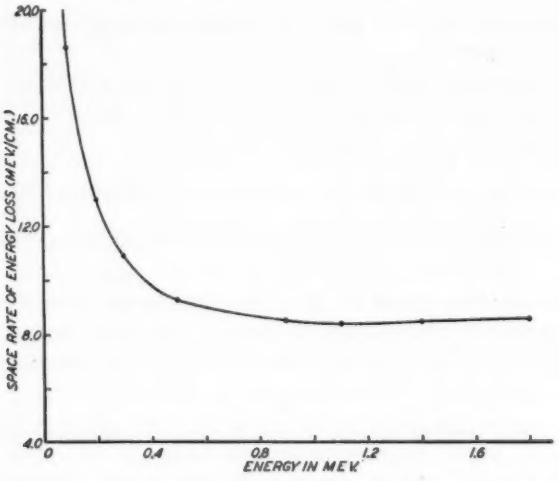

FIG. 7. Relation between the total space rate of β-particle energy loss (radiation and ionization) in a silver bromide medium and the kinetic energy of that particle.

(0.05 mev.), I^{131} (0.2 mev.), and P^{32} (0.7 mev.), that the photographic efficiency for the emitted β-particles in the initial part of their path decreases in that order while the range of the β-particles increases respectively.

Using these data, it has been possible to estimate the theoretical photographic efficiency for I^{131} β-particles acting on medium contrast emulsion.

From the β-ray spectrum of I^{131} and the relation between $-\left(\frac{dT}{dx}\right)$ and T, the mean $-\left(\frac{dT}{dx}\right)$ for I^{131} over the range of T was graphically estimated to be 15.7 mev. per cm.

The average diameter of an undeveloped silver bromide grain (E.K. medium contrast lantern slide) was experimentally determined as 0.4 μ. The mean grain distance traversed by a β-particle through a series of assumedly spherical grains, which can be shown to be 0.64 times the average diameter, is for this type of grain 0.25 μ. This assumes, of course, a linear pathway, the effect of scattering being neglected.

The mean energy expended by a β-particle immediately ejected from I^{131} within the emulsion grain is, therefore, theoretically evaluated as:

$$(15.7 \times 10^6) \times (0.25 \times 10^{-4}) = 390 \text{ ev.}$$

The wave length of a photon containing an equivalent amount of energy is 32 Å (X ray). The basic assumption is made that the energy dissipated by a β-particle, in its path through an emulsion grain, has a photochemical effect (i.e., in the production of a latent image) equivalent to that produced by the absorption of an X-ray photon with identical energy content. One quantum of energy at this range of wave length has been estimated on the basis of experimental results to cause the deposition of eight reduced silver atoms within the emulsion grain (6). The validity of this finding was substantiated by Glocker and Traub (4).

The next consideration is the number of deposited silver atoms required to render a grain developable. Webb (9) has statistically determined that on the average 16–130 quanta of light (wave length 4000 Å) are absorbed per grain to produce a density range of 1.0–3.0 in medium contrast photographic emulsion. Since one quantum with wave length 4000 Å contains approximately 3 ev., the mean energy required to render a medium contrast emulsion grain developable is about 48–390 ev. in this range of densities. An average of 5 ev. (3) are spent in bringing an electron into the conduction band of the silver bromide molecule, and, subsequently causing the deposition of one silver atom. Hence, by division, 10–78 silver atoms are deposited per medium grain to produce the selected density range.

Now, since it has been evaluated that one β-particle at the commencement of its course will on the average cause the deposition of eight reduced silver atoms within the medium contrast lantern slide grain, it follows, therefore, that about 2–10 impacts are required to develop a grain. This is a theoretical substantiation of previously estimated results (8). In view of the assumptions made throughout this theory, the results are interpreted as supplying at most only an order of efficiency.

The theoretical aspect of the geometrical relations of the radioactive source and emulsion has served to illustrate the practical conditions for optimum resolution. Furthermore, since photographic density is affected by the geometry of the autographic preparation, these results can be employed in quantitative autography to account for variations in the shape and size of the microscopic sources of radioactivity. With the medium contrast photographic emulsions used in practice it is readily observed that tracks are scarce. This observation has been explained by the theoretical finding that, on the average, more than one impact per grain is required by the beta particle in order to render it developable.

Acknowledgments

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INHIBITORS OF THE β-GLUCURONIDASE OF RABBIT POLYMORPHONUCLEAR LEUCOCYTES[1]

By Esther Wong and R. J. Rossiter

Abstract

As previously reported, rabbit polymorphonuclear leucocytes contain an enzyme capable of hydrolyzing biosynthetic phenolphthalein mono-β-glucuronide. The effect of a number of inhibitors on cell-free preparations of this enzyme was studied. Many of the usual enzyme inhibitors were without effect, but certain dicarboxylic acids were strong inhibitors. These were (in order of activity): D-glucosaccharic acid, D-mucic acid, L-malic acid, mesotartaric acid, DL-malic acid, and L-tartaric acid. The nature of the inhibition was competitive. The Michaelis constant, K_s, was evaluated for phenolphthalein mono-β-glucuronide and values of K_i were estimated for glucosaccharic acid, mucic acid, DL-malic acid, and mesotartaric acid. Heparin in high concentration was slightly inhibitory, but the nature of the inhibition was noncompetitive. For each of the inhibitors studied, the extent of the inhibition was influenced by the hydrogen ion concentration. At a pH greater than 4.5, the optimum for the enzyme under the conditions used, the degree of inhibition was less, and at a pH less than 4.5 the degree of inhibition was greater.

Introduction

Rabbit polymorphonuclear leucocytes contain a β-glucuronidase capable of hydrolyzing phenolphthalein mono-β-glucuronide (Rossiter and Wong (12)). This enzyme is also present in the white cells of human blood (1, 5, 13). As early as 1936, Oshima (11) showed that the hydrolysis of menthylglucuronide by ox spleen β-glucuronidase was inhibited by certain dicarboxylic acids, notably, malic acid, tartaric acid, and citric acid. More recently, Karunairatnam and Levvy (7) studied the effect of a number of inhibitors on the hydrolysis of phenylglucuronide by mouse-liver β-glucuronidase.

In the present paper the effect of a number of inhibitors on the β-glucuronidase of the polymorphonuclear leucocyte of the rabbit is described. These inhibitors include a series of dicarboxylic acids and a number of the well known enzyme poisons.

Methods

Enzyme Preparation

Polymorphonuclear leucocytes were obtained from the rabbit by the method of de Haan (6), the details of which have already been described by Cram and Rossiter (4). From these cells a soluble enzyme was prepared in saponin, as described by Rossiter and Wong (12).

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Contribution from the Department of Biochemistry, University of Western Ontario, London, Ont. The work was supported by grants from the National Cancer Institute and National Research Council of Canada.

β-Glucuronidase Determination

The glucuronidase activity was determined by the method of Fishman, Springer, and Brunetti (5), using the biosynthetic phenolphthalein mono-β-glucuronide, prepared as described by these workers. For work with inhibitors it was found desirable to modify the procedure in several respects.

To each of a series of centrifuge tubes was added:—

1.2 ml. 0.1 *M* acetate buffer pH 4.5.

0.1 ml. phenolphthalein mono-β-glucuronide solution.

The concentration of this solution varied from 0.01 *M* to 0.000156 *M*.

0.5 ml. enzyme solution.

0.2 ml. inhibitor dissolved in 0.1 *M* acetate buffer, pH 4.5. If necessary, this solution was titrated to a final pH of 4.5.

A similar series of tubes, in which the inhibitor was replaced by 0.2 ml. buffer, and a series of control tubes, containing enzyme and buffer but no substrate, were also set up. The tubes were placed in a 38° C. water bath for 12–16 hr., at the end of which time the reaction was stopped by the addition of 1 ml. 10% trichloroacetic acid. Inhibitor (0.2 ml.) was then added to all tubes from which it had previously been omitted and buffer (0.2 ml.) was added to the remaining tubes. Substrate (0.1 ml.) was added to the control tubes. Each tube was then titrated with 1.0 *N* sodium hydroxide to the pink color of phenolphthalein. The color was discharged with one drop of 1.0 *N* hydrochloric acid and then 0.1 *N* sodium hydroxide was carefully added to the first permanent pink of phenolphthalein. The tubes were centrifuged and the supernatant decanted through small funnels containing cotton plugs into graduated tubes containing 2.5 ml. 0.04 *M* glycine buffer. The precipitate was washed with small quantities of water and the final volume of the supernatant and washings was made up to 8 ml. The color density was then read against a blank in a Coleman universal spectrophotometer at 540 mμ. A standard containing 20 μgm. phenolphthalein per tube was neutralized as described for the tests, diluted with 2.5 ml. glycine buffer, made up to 8 ml., and read with each set of determinations. Experiments were always run in duplicate.

Recording of Results

Enzyme activity is recorded as the number of μgm. phenolphthalein liberated per 100 ml. enzyme solution per hour from the phenolphthalein mono-β-glucuronide under the standard conditions of the test.

Results

As anticipated from the results of Karunairatnam and Levvy (7) with mouse-liver β-glucuronidase, D-glucosaccharic acid* was the most effective

* *Kindly provided by Dr. F. L. M. Pattison, Department of Chemistry.*

inhibitor of the rabbit polymorphonuclear leucocyte enzyme. A concentration of 5×10^{-3} *M* caused almost complete inhibition and concentrations as low as 10^{-5} *M* produced a degree of inhibition that was quite measurable (Table I).

TABLE I

INHIBITION OF β-GLUCURONIDASE OF RABBIT POLYMORPHONUCLEAR LEUCOCYTES BY D-GLUCOSACCHARIC ACID. SUBSTRATE, PHENOLPHTHALEIN MONO-β-GLUCURONIDE (0.0005 *M*). TEMPERATURE, 38° C. pH, 4.5

Glucosaccharic acid (*M*)	Preparation 1		Preparation 2	
	Enzyme activity, μgm. phenolphth./ 100 ml./hr.	Percentage inhibition	Enzyme activity, μgm. phenolphth./ 100 ml./hr.	Percentage inhibition
0	1800	—	666	—
10^{-6}	1790	1	663	1
10^{-5}	1660	8	625	6
10^{-4}	1100	39	392	41
10^{-3}	248	86	106	84
$10^{-2} \times 0.5$	73	96	14	98

Other inhibitors were much less effective. D-Mucic acid in a concentration of 10^{-3} *M* caused an order of inhibition similar to that of D-glucosaccharic acid in a concentration of 10^{-4} *M* (Table II). D-Glucuronic acid lactone, L-malic acid, DL-malic acid, L-tartaric acid, and mesotartaric acid were all inhibitory (Table II), but concentrations of 10^{-2} *M* were necessary to produce a degree of inhibition comparable to that of 10^{-4} *M* D-glucosaccharic acid or 10^{-3} *M* D-mucic acid.

Becker and Friedenwald (2) reported that heparin in concentrations from 0.4 to 40 mgm. per 100 ml. caused an inhibition of the order of 50% in the rate of hydrolysis of phenolphthalein mono-β-glucuronide by the β-glucuronidase of bovine aqueous humor and other sources. Table II shows that heparin in a final concentration of 40 mgm. per 100 ml., or 38 Toronto units per ml., inhibited the rabbit polymorphonuclear leucocyte enzyme only to the extent of 20%. Heparin in a concentration of 4 mgm. per 100 ml. and 0.4 mgm. per 100 ml. was without effect.

The following substances in a final concentration of 10^{-2} *M* produced negligible (less than 10%) inhibition of the rabbit polymorphonuclear leucocyte enzyme: D-glucose, D-glucosamine, D-gluconic acid,* adipic acid, ascorbic acid, citric acid, oxalic acid, succinic acid, malonic acid, lactic acid, acetic acid, propionic acid, butyric acid, boric acid, phosphoric acid, iodoacetic acid, sodium cyanide, sodium azide, sodium fluoride, glycine, thiourea, urethane, phenylacetic acid, *o*-cresol, *m*-cresol, salicylic acid, arsanilic acid, tri-*o*-cresyl phosphate, and tri-*m*-cresyl phosphate.

* *Kindly provided by Dr. F. L. M. Pattison, Department of Chemistry.*

TABLE II

EFFECT OF INHIBITORS ON β-GLUCURONIDASE OF RABBIT POLYMORPHONUCLEAR LEUCOCYTES. SUBSTRATE, PHENOLPHTHALEIN MONO-β-GLUCURONIDE (0.0005 *M*). TEMPERATURE, 38° C. pH, 4.5

Inhibitor	Concentration (*M*)	Enzyme activity, μgm. phenolphthalein /100 ml./hr.		Percentage inhibition
		No inhibitor	With inhibitor	
D-Glucosaccharic acid	10^{-4}	350	220	37
		363	215	41
		666	392	41
		1080	639	41
		1800	1100	39
D-Mucic acid	10^{-3}	575	384	33
		710	464	35
		1080	702	35
D-Glucuronic acid lactone	10^{-2}	547	472	14
		996	800	20
L-Malic acid	10^{-2}	1255	480	62
		1250	493	60
DL-Malic acid	10^{-2}	459	262	43
		678	415	39
		610	363	40
		1220	630	48
		1260	633	50
L-Tartaric acid	10^{-2}	750	630	16
		1245	1060	15
		1255	1085	14
Mesotartaric acid	10^{-2}	1260	518	59
		1260	510	59
		1360	653	52
Heparin	40 mgm./ 100 ml.	510	391	23
		2180	1770	19

Nature of Inhibition

According to the classical Michaelis–Menten theory the relation between the initial velocity of an enzyme reaction and the concentration of the substrate can be expressed as:

$$\frac{1}{V} = \frac{K_s}{V_m}\left(\frac{1}{S}\right) + \frac{1}{V_m} \qquad (1)$$

where V is the initial velocity of the reaction, S is the concentration of substrate, V_m the maximum initial velocity at the optimum substrate concentration, and K_s is the Michaelis constant i.e., the dissociation constant of a hypothetical enzyme–substrate complex.

If $\frac{1}{V}$ is plotted against $\frac{1}{S}$, as described by Lineweaver and Burk (8), a straight line results with an intercept equal to $\frac{1}{V_m}$ and the slope equal to $\frac{K_s}{V_m}$. Fig. 3 of the previous paper by Rossiter and Wong (12) showed that the enzyme–substrate relations of the rabbit polymorphonuclear leucocyte β-glucuronidase could be described in terms of the Michaelis–Menten theory.

In the presence of a competitive inhibitor, the above equation becomes:

$$\frac{1}{V} = \frac{K_s}{V_m}\left(1 + \frac{(I)}{K_i}\right)\frac{1}{S} + \frac{1}{V_m} \qquad (2)$$

where V is the initial velocity of the reaction in the presence of the inhibitor at a concentration (I), V_m is the maximum initial velocity in the presence of the inhibitor, K_i is the dissociation constant of a hypothetical enzyme–inhibitor complex, and the other symbols have the same meaning as before. With a competitive inhibitor, the maximum initial velocity, V_m, is the same whether the inhibitor is present or not. If $\frac{1}{V}$ is plotted against $\frac{1}{S}$ a straight line still results with the intercept equal to $\frac{1}{V_m}$, but the slope, previously $\frac{K_s}{V_m}$, is now multiplied by the factor $\left(1 + \frac{(I)}{K_i}\right)$. This is characteristic of competitive inhibition.

If the inhibition is noncompetitive,

$$\frac{1}{V} = \frac{K_s}{V_m}\left(1 + \frac{(I)}{K_i}\right)\frac{1}{S} + \left(1 + \frac{(I)}{K_i}\right)\frac{1}{V_m} \qquad (3)$$

i.e. both the slope, formerly $\frac{K_s}{V_m}$, and the intercept, formerly $\frac{1}{V_m}$, are multiplied by the factor $\left(1 + \frac{(I)}{K_i}\right)$.

Table III shows the results of typical experiments with D-glucosaccharic acid (10^{-4} M) and with D-mucic acid (10^{-3} M). Similar experiments with DL-malic acid (10^{-2} M) and mesotartaric acid (10^{-2} M) are given in Table IV. In each of these instances $\frac{1}{V}$ was plotted against $\frac{1}{S}$ (Figs. 1–4). The lines shown in the figures are the calculated lines of best fit.

In the presence of the inhibitor, although the slope of the curve increased, the intercept remained the same, indicating that, for each inhibitor studied, the inhibition was competitive.

From the slope and intercept of the uninhibited curves of Figs. 1–4, four different estimates of K_s were obtained graphically. These values for the Michaelis constant of β-glucuronidase with phenolphthalein mono-β-glucuronide as substrate, at a pH of 4.5 and at a temperature of 38° C., 0.85×10^{-4} M, 0.90×10^{-4} M, 0.87×10^{-4} M, 0.96×10^{-4} M, can be compared with our previously published figures, which ranged from 0.93×10^{-4} M to 1.17×10^{-4} M (Rossiter and Wong (12)).

TABLE III

EFFECT OF SUBSTRATE CONCENTRATION ON THE INHIBITION OF THE β-GLUCURONIDASE OF RABBIT POLYMORPHONUCLEAR LEUCOCYTES BY D-GLUCOSACCHARIC ACID (10^{-4} *M*) AND D-MUCIC ACID (10^{-3} *M*). SUBSTRATE CONCENTRATION, *S*, IN GM-MOL. PER LITER. TEMPERATURE 38° C. pH, 4.5

$S \times 10^5$	Initial velocity, *V*, (μgm phenolphthalein/100 ml./hr.)			
	D-Glucosaccharic acid (10^{-4} *M*)		D-Mucic acid (10^{-3} *M*)	
	No inhibitor	With inhibitor	No inhibitor	With inhibitor
7.8	199	54	406	109
15.6	255	95	513	181
31.2	318	149	623	300
62.5	350	220	710	464
125	380	287	855	685
250	433	404	905	838
Mean deviation of *V* from calc.	11	13	42	29
V_m	408	422	855	944
K_s	0.85×10^{-4}	—	0.90×10^{-4}	—
$K_s\left(1+\frac{(I)}{K_i}\right)$	—	0.54×10^{-3}	—	0.60×10^{-3}
K_i	—	0.18×10^{-4}	—	0.18×10^{-3}

TABLE IV

EFFECT OF SUBSTRATE CONCENTRATION ON THE INHIBITION OF THE β-GLUCURONIDASE OF RABBIT POLYMORPHONUCLEAR LEUCOCYTES BY DL-MALIC ACID (10^{-3} *M*) AND MESOTARTARIC ACID (10^{-3} *M*). SUBSTRATE CONCENTRATION, *S*, IN GM-MOL. PER LITER. TEMPERATURE, 38° C. pH, 4.5.

$S \times 10^5$	Initial velocity, *V*, (μgm. phenolphthalein/100 ml./hr.)			
	DL-Malic acid (10^{-3} *M*)		Mesotartaric acid (10^{-3} *M*)	
	No inhibitor	With inhibitor	No inhibitor	With inhibitor
7.8	377	97	725	141
15.6	494	174	969	234
31.2	588	286	1172	397
62.5	678	415	1360	653
125	755	582	1500	988
250	780	691	1634	1345
Mean deviation of *V* from calc.	16	8	36	72
V_m	786	834	1600	1410
K_s	0.87×10^{-4}	—	0.96×10^{-4}	—
$K_s\left(1+\frac{(I)}{K_i}\right)$	—	0.59×10^{-3}	—	0.72×10^{-3}
K_i	—	0.17×10^{-3}	—	0.15×10^{-3}

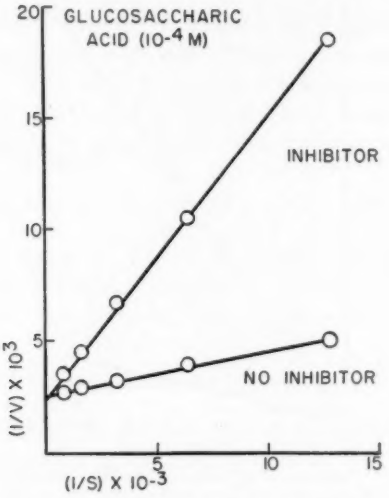


FIG. 1. Plot of $1/V$ against $1/S$ for rabbit polymorphonuclear leucocyte β-glucuronidase in presence or absence of D-glucosaccharic acid 10^{-4} M. V is the initial velocity in μgm. phenolphthalein per 100 ml. per hr. and S is the substrate concentration in gm-mol. per liter.

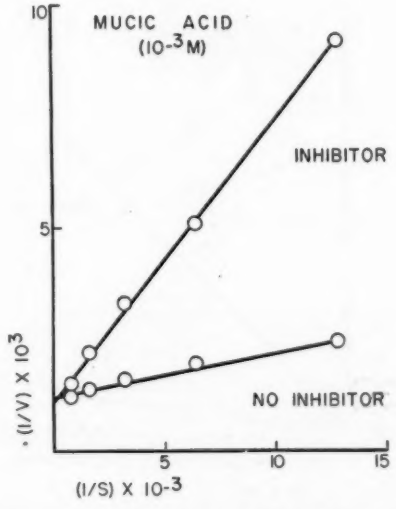


FIG. 2. Plot of $1/V$ against $1/S$ for rabbit polymorphonuclear leucocyte β-glucuronidase in presence or absence of D-mucic acid 10^{-3} M. V is the initial velocity in μgm phenolphthalein per 100 ml. per hr. and S is the substrate concentration in m-mol. per liter.

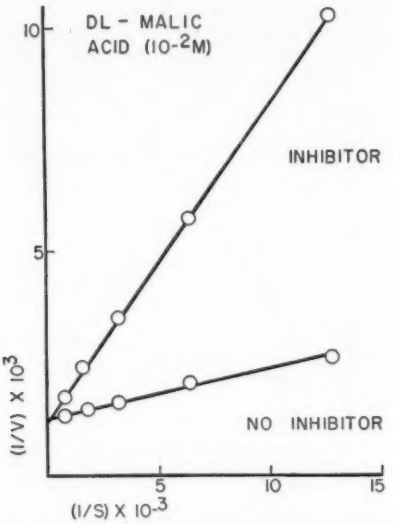


FIG. 3. Plot of $1/V$ against $1/S$ for rabbit polymorphonuclear leucocyte β-glucuronidase in presence or absence of DL-malic acid 10^{-2} M. V is the initial velocity in μgm. phenolphthalein per 100 ml. per hr. and S is the substrate concentration in gm-mol. per liter.

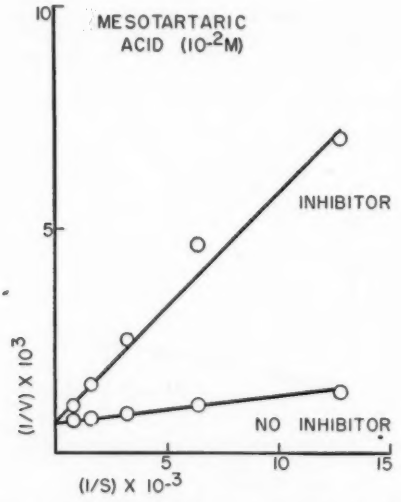


FIG. 4. Plot of $1/V$ against $1/S$ for rabbit polymorphonuclear leucocyte β-glucuronidase in presence or absence of mesotartaric acid 10^{-2} M. V is the initial velocity in μgm. phenolphthalein per 100 ml. per hr. and S is the substrate concentration in gm-mol. per liter.

For each inhibitor an estimate of $K_s\left(1 + \frac{(I)}{K_i}\right)$ was obtained graphically and from this was calculated the value of K_i. The values obtained, 0.18×10^{-4} *M* for D-glucosaccharic acid, 0.18×10^{-3} *M* for D-mucic acid, 0.17×10^{-2} *M* for DL-malic acid, and 0.15×10^{-2} *M* for mesotartaric acid, indicate the relative effectiveness of these inhibitors and confirm the findings presented in Table II.

In order to obtain an estimate of the accuracy of the experimental data, the initial velocity, *V*, was calculated for each concentration of substrate by substituting the values of K_s, V_m, K_i determined graphically in either Equation (1) for the uninhibited enzyme or Equation (2) for the inhibited enzyme. The mean deviations of the experimental from the calculated values of *V* are given in Tables III and IV.

The inhibition with heparin (40 mgm. per 100 ml.) was also studied. Fig. 5 shows the plot of $\frac{1}{S}$ against $\frac{1}{V}$ for a typical experiment. In the presence of the inhibitor both the slope and the intercept increased, indicating that there was a strong noncompetitive element in the inhibition.

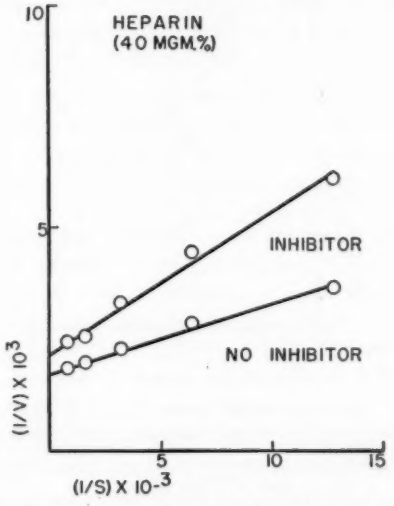


FIG. 5. Plot of $1/V$ against $1/S$ for rabbit polymorphonuclear leucocyte β-glucuronidase in presence or absence of heparin 40 mgm. per 100 ml. *V* is the initial velocity in μgm. phenolphthalein per 100 ml. per hr. and *S* is the substrate concentration in gm-mol. per liter.

Effect of Hydrogen Ion Concentration on the Degree of Inhibition

The pH optimum for rabbit polymorphonuclear leucocyte β-glucuronidase with phenolphthalein mono-β-glucuronide as substrate was found to be 4.5 (12). Mills and co-workers (9, 10) claimed that ox spleen contained at least

three glucuronidases, each with a different pH optimum. A contrary view was expressed by Bernfeld and Fishman (3), who stated the belief that the pH optimum of the spleen enzyme was 4.5 and that the pH optima of the different fractions obtained by Mills might be due to the presence of contaminating inhibitors. In this regard it is of interest to note that the pH optimum of an electrophoretically homogeneous calf glucuronidase was shifted from 4.5 to over 5 by the addition of substances such as saccharic acid or gluconic acid. Since Mills (10) suggested that his preparations differed in their sensitivity to certain inhibitors, the effect of the hydrogen ion concentration on the inhibition of the rabbit polymorphonuclear leucocyte β-glucuronidase was studied.

Fig. 6 shows that for each of the inhibitors studied, D-glucosaccharic acid, D-mucic acid, DL-malic acid, and heparin, the extent of the inhibition was greater at pH 3.4 than at pH 4.5 and greater at pH 4.5 than at pH 5.2. A greater degree of inhibition was thus observed at a pH that was lower than the optimal pH for the enzyme in the absence of the inhibitor.

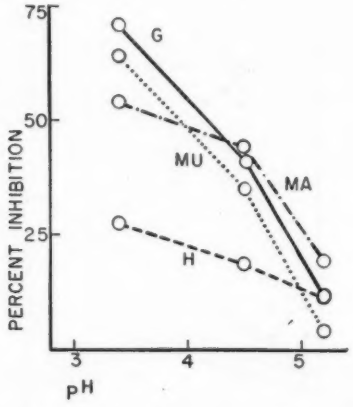


FIG. 6. The effect of pH on the degree of inhibition of rabbit polymorphonuclear leucocyte β-glucuronidase by D-glucosaccharic acid 10^{-4} *M* (G), D-mucic acid 10^{-3} *M* (MU), DL-malic acid 10^{-2} *M* (MA), and heparin 40 mgm. per 100 ml. (H).

Discussion

A number of the common enzyme poisons did not inhibit the rabbit polymorphonuclear leucocyte β-glucuronidase. The following substances produced negligible inhibition when present in the reaction mixture in a final concentration of 0.01 *M*: iodoacetate, cyanide, fluoride, azide, oxalate, malonate urethane, thiourea, arsanilic acid, borate, phosphate, glycine, phenylacetate, salicylate, *o*-cresol, *m*-cresol, tri-*o*-cresyl phosphate, tri-*m*-cresyl phosphate,

and acetophenone. Of these substances, fluoride, oxalate, malonate, urethane, and thiourea, in a concentration of 0.015 *M*, have been shown to have no effect on mouse-liver β-glucuronidase (7).

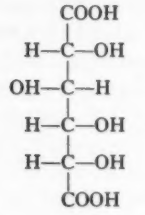
The observation that the most effective inhibitor of rabbit polymorphonuclear leucocyte β-glucuronidase was D-glucosaccharic acid and that the inhibition due to this substance was of a competitive nature agrees with the findings of Karunairatnam and Levvy (7) for mouse liver and Mills (10) for ox spleen. The value of 0.18×10^{-4} *M*, found for the K_i of D-glucosaccharic acid, is of the same order as the value of 0.6×10^{-4} *M* reported by Karunairatnam and Levvy (7). Since these workers were working at a pH of 5.2, rather than at 4.5, their value would be expected to be greater. As Karunairatnam and Levvy (7) pointed out, the accuracy of the determination of K_i depends upon the validity of the estimate of K_s, which may vary with different preparations of the enzyme. With eight preparations of rabbit white cell enzyme we obtained a mean value for K_s (± S.D.) of $0.97 \times 10^{-4} \pm 0.10 \times 10^{-4}$.

The relative degree of inhibition observed with the dicarboxylic acids, D-glucosaccharic acid, D-mucic acid, L-malic acid, and DL-malic acid, is similar to that reported by Karunairatnam and Levvy (7) for mouse-liver glucuronidase. These workers, however, reported a slight degree of inhibition with D-gluconic acid and a degree of inhibition with D-glucuronic acid lactone not observed for the rabbit white cell enzyme.

Taking D-glucosaccharic acid (I) as a pattern, an attempt was made to correlate the chemical structure of the inhibitor with its inhibitory powers. Apparently for the 6-carbon compounds a carboxyl group at both carbon-1 and carbon-6 is essential. Of all the other compounds with six carbon atoms tested, only the dicarboxylic acid, D-mucic acid (II), was inhibitory, although it was a much weaker inhibitor than D-glucosaccharic acid. D-Gluconic acid (III), with a structure similar to D-glucosaccharic acid except that the carboxyl group at carbon-6 is replaced by a primary alcohol group, D-glucuronic acid lactone (IV) in which the carboxyl group at carbon-1 is replaced by an aldehyde group, and D-glucose (V), in which both the carboxyl groups are replaced, caused negligible inhibition. Shorter chain dicarboxylic acids, such as malic acid (VII), L-tartaric acid (VIII), and mesotartaric acid (IX), were inhibitory, but they were much less active than either D-glucosaccharic acid or D-mucic acid. Monocarboxylic acids, such as acetic acid, propionic acid, and butyric acid, and even hydroxy-monocarboxylic acids, such as lactic acid, were without effect.

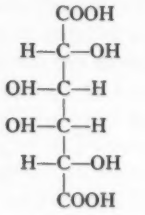
It is clear, however, that a carboxyl group at each end of the molecule is not all that is required to produce an inhibition, since adipic acid (VI), as well as succinic acid, malonic acid, and oxalic acid, were all inactive. A hint as to what groupings might be involved in the inhibitory action is given by the finding that D-mucic acid (II) is a much weaker inhibitor than D-glucosaccharic acid (I). The difference between the chemical structure of these two molecules is in the spatial configuration of the hydroxyl group at carbon-4. It will be

noted that all of the substances that produced an inhibition (I, II, VII, VIII, IX) contain an hydroxyl group attached to a carbon atom in the β-position relative to one of the carboxyl groups. The importance of the spatial configuration of the hydroxyl group on either the α- or β-carbon atom is shown by the finding that L-malic acid was a stronger inhibitor than DL-malic acid



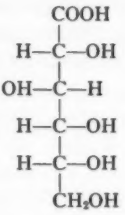
D-Glucosaccharic acid

I



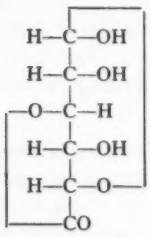
D-Mucic acid

II



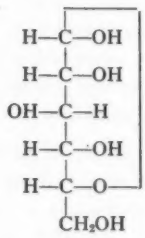
D-Gluconic acid

III



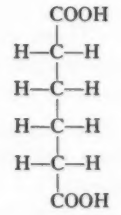
D-Glucuronic acid Lactone

IV



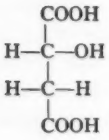
D-Glucose

V



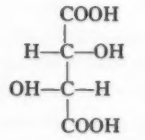
Adipic acid

VI



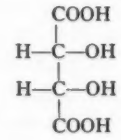
L-Malic acid

VII



L-Tartaric acid

VIII



Mesotartaric acid

IX

in the same concentration. Presumably the D-isomer is a less effective inhibitor. Additional evidence for the importance of the spatial configuration of such an hydroxyl group is the finding that mesotartaric acid (IX) was a stronger inhibitor than L-tartaric acid (VIII). D-Tartaric was not available.

An insufficient number of compounds were available to characterize the chemical groupings necessary to produce an inhibition. A complete analysis of the problem would involve the testing of a variety of rather inaccessible

compounds. On the limited evidence presented, a generalization would appear to be that the requirements for the competitive inhibition of β-glucuronidase are an α,ω-dicarboxylic acid with a favorable spatial distribution of hydroxyl groups attached to the intervening carbon atoms.

The position of ascorbic acid and heparin as inhibitors of β-glucuronidase is far from clear. Becker and Friedenwald (2) found that 20 mgm. ascorbic acid per 100 ml. inhibited the β-glucuronidase of bovine aqueous humor to the extent of 80%. This represents the concentration of just over 10^{-3} *M*, whereas, in our experiments with the rabbit polymorphonuclear leucocyte enzyme, 10^{-2} *M* ascorbic acid produced negligible inhibition. Becker and Friedenwald (2) also found that heparin in concentrations of 0.4 mgm. to 40 mgm. per 100 ml. inhibited aqueous humor β-glucuronidase to the extent of 50%. With the white cell enzyme, heparin, in concentrations of 0.4 mgm. and 4.0 mgm. per 100 ml., was without effect and 40 mgm. per 100 ml. inhibited to the extent of only 20%. This latter concentration corresponds to 38 Toronto units per ml. The observation that heparin in a concentration of 4 mgm. per 100 ml., i.e. 3.8 Toronto units per ml., was not inhibitory agrees with the report of Karunairatnam and Levvy (7), who found that the mouse-liver glucuronidase was not inhibited by 6.6 Toronto units per ml. The reason for the difference between our results and those of Becker and Friedenwald (2) may be that the enzyme preparations were of different purity, or it may be that there is a genuine difference in the properties of enzymes obtained from different sources. Limitation of the amount of material available has precluded any attempts at purifying the white cell enzyme preparations.

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ENZYMES OF GRAY MATTER AND WHITE MATTER OF DOG BRAIN

THE DISTRIBUTION OF CERTAIN NONOXIDATIVE ENZYMES[1]

By A. R. McNabb[2]

Abstract

Observations were made on the activity of the cholinesterases (hydrolysis of acetylcholine, acetyl-β-methylcholine, and benzoylcholine), common esterase (hydrolysis of tributyrin in the presence of 10^{-5} *M* eserine), acid and alkaline phosphomonoesterases (hydrolysis of disodium phenyl phosphate at pH 4.9 and pH 9.9–10), amylase (hydrolysis of soluble starch and glycogen) and β-glucuronidase (hydrolysis of phenolphthalein mono-β-glucuronide) in the gray matter and the white matter of the brains of a series of dogs. With the exception of the β-glucuronidase, the concentration of each of these enzymes, when expressed on a wet weight basis, was greater in the gray matter than in the white matter, although the relative concentrations differed for each enzyme.

Introduction

Previous work from this laboratory has been concerned with the lipids of the gray matter and the white matter of brain (18, 19). Gray matter, which contains many nerve cell bodies and is relatively poor in myelin sheath material, would be expected to differ in its enzyme content from white matter, which contains much fewer nerve cells and is relatively rich in myelin sheaths. This expectation is borne out by the finding that gray matter has a higher oxygen consumption (7, 15), glycolytic rate (15), and indophenol oxidase activity (15) than white matter. Gray matter also contains a higher concentration of substances such as cytochrome (15), thiamine (41), riboflavin (13), and iron (16) that are intimately linked with the metabolic process. However, reports on the distribution of nonoxidative enzymes are somewhat fragmentary and, in a number of instances, they are even conflicting.

This paper is an attempt to bring together a number of these isolated reports on nonoxidative enzymes and to present results obtained for other enzymes that have hitherto not been investigated from this point of view. Observations have been made on cholinesterases (hydrolysis of acetylcholine, acetyl-β-methylcholine and benzoylcholine), common esterase (ali-esterase), phosphomonoesterases (acid and alkaline), amylase, and β-glucuronidase.

Methods

Adult dogs were anaesthetized with chloroform, exsanguinated, and the brains removed as quickly as possible. Samples of tissue (about 2 gm.) were taken from gray matter and white matter, rapidly weighed, and homogenized

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Contribution from the Department of Biochemistry, University of Western Ontario, London, Ont.

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in 10 ml. 0.9% sodium chloride with an ice-cooled Potter–Elvehjem type glass homogenizer. Until they were used the homogenates were kept in an ice bath. The sample of gray matter consisted of thin shavings of cortex from the cerebral hemispheres. The sample of white matter was chiefly corpus callosum. Additional samples of both gray matter and white matter were also taken for the determination of the wet weight to dry weight ratios by heating to constant weight at 105° C.

Enzyme Determinations

For each of the determinations 0.2 ml. of the homogenate was taken except for the phosphomonoesterase estimations where it was necessary to dilute the homogenates by a factor of 50. All determinations and the appropriate blanks were run in duplicate.

Cholinesterases

Cholinesterase activity was determined by the microtitration procedure described by Colling and Rossiter (5). The final concentrations of substrate were: acetylcholine 0.006 *M*, acetyl-β-methylcholine 0.03 *M*, and benzoylcholine 0.006 *M*. The incubation time was four hours. Under the conditions of the experiment this concentration of substrate was optimal for acetyl-β-methylcholine and benzoylcholine and less than 10% of the substrate was hydrolyzed. For acetylcholine the optimal substrate concentration depends upon the relative concentrations of true cholinesterase and pseudocholinesterase in the preparation. As in previous work on the cerebrospinal fluid (5) a substrate concentration of 0.006 *M* was arbitrarily selected for acetylcholine. Results are reported in terms of ml. 0.001 *N* acid liberated from 100 mgm. brain tissue per hour. According to Mendel, Mundell, and Rudney (23), the hydrolysis of acetyl-β-methylcholine is a measure of the true cholinesterase (24), specific cholinesterase (29), *e*-type cholinesterase (43), acetylcholinesterase (3), or acetocholinesterase (38), and the hydrolysis of benzoylcholine is a measure of the pseudocholinesterase (24), nonspecific cholinesterase (29), *s*-type cholinesterase (43), or butyrocholinesterase (38).

Common Esterase

Common esterase (ali-esterase of Richter and Croft (33)) was determined as described above, using tributyrin 0.007 *M* as substrate. Since it is now well known that tributyrin is hydrolyzed by pseudocholinesterase (2, 24, 29), the pseudocholinesterase activity was suppressed by the addition of eserine in a final concentration of 10^{-5} *M* (8, 24, 26, 33, 38). Results are reported in terms of ml. 0.001 *N* acid liberated by 100 mgm. brain tissue per hour.

Phosphomonoesterases

Phosphomonoesterase activity was determined by the modification of the original method of King and Armstrong (20) described by Colling and Rossiter (6). Acid phosphomonoesterase was determined in citrate buffer pH 4.9 (42) and alkaline phosphomonoesterase in carbonate–bicarbonate buffer pH

9.9–10 (21). The reaction mixture contained magnesium chloride in a final concentration of 0.001 *M*, a concentration that is optimal for many phosphatases (17). The incubation time was one hour. Results are reported in terms of the amount of phenol (in mgm.) liberated from the substrate disodium phenyl phosphate (0.004 *M*) per 100 mgm. brain tissue per hour.

Amylase

Amylase activity was determined by a modification of the method of Somogyi (37). The reducing sugar liberated from 0.14% soluble starch or 0.14% glycogen was determined by the colorimetric method of Nelson (30). The incubation time was four hours. Results are reported in terms of the amount of reducing sugar as glucose (in μgm.) liberated from either the starch or glycogen per 100 mgm. brain tissue per hour.

β-Glucuronidase

β-Glucuronidase was measured by the method of Fishman, Springer, and Brunetti (11) using the biosynthetic substrate, phenolphthalein mono-β-glucuronide, prepared as described by these workers. The details of the procedure have been described by Rossiter and Wong (34). The incubation time was three hours. Results are reported as the amount of phenolphthalein (in μgm.) liberated from the phenolphthalein mono-β-glucuronide (0.001 *M*) per 100 mgm. brain tissue per hour.

Results

Table I shows the mean (± S.E. mean) values for the cholinesterases. On a wet weight basis there was a greater hydrolysis of acetylcholine by gray matter than by white matter. As was expected, the brain hydrolyzed acetyl-β-methylcholine more rapidly than benzoylcholine, but the relative rates of hydrolysis of these two substrates was of the same order in both gray matter and white matter, i.e. there was little difference between the ratio of the concentration of true cholinesterase to that of pseudocholinesterase in the two types of brain tissue.

TABLE I

CHOLINESTERASE ACTIVITY OF GRAY MATTER AND WHITE MATTER OF DOG BRAIN (ML. 0.001 *N* ACID PER 100 MGM. WET WEIGHT PER HR.)

	Acetylcholine, 0.006 *M*			Acetyl-β-methylcholine, 0.03 *M*			Benzoylcholine, 0.006 *M*		
	No.	Mean	S.E. of mean	No.	Mean	S.E. of mean	No.	Mean	S.E. of mean
Gray matter	8	4.12	±0.17	8	3.09	±0.46	8	0.98	±0.13
White matter	8	3.04	±0.32	7	2.00	±0.24	8	0.58	±0.11
Gray matter : white matter ratio	1.4 : 1			1.5 : 1			1.7 : 1		

The results for common esterase and the phosphomonoesterases are given in Table II. On a wet weight basis the activity of each enzyme in the gray matter was approximately twice that in the white matter. The ratio of the activity of the gray matter to that of the white matter was a little greater than that observed for the cholinesterases.

TABLE II

COMMON ESTERASE (ML. 0.001 *N* ACID PER 100 MGM. WET WEIGHT PER HR.) ACTIVITY AND ACID AND ALKALINE PHOSPHOMONOESTERASE (MGM. PHENOL PER 100 MGM. PER HR.) ACTIVITY OF GRAY MATTER AND WHITE MATTER OF DOG BRAIN

	Common esterase (tributyrin, 0.007 *M*)			Acid phosphomonoesterase (pH 4.9)			Alkaline phosphomonoesterase (pH 9.9–10)		
	No.	Mean	S.E. of mean	No.	Mean	S.E. of mean	No.	Mean	S.E. of mean
Gray matter	5	4.54	±0.40	8	0.48	±0.04	8	1.97	±0.27
White matter	5	2.40	±0.52	8	0.23	±0.04	8	1.02	±0.17
Gray matter : white matter ratio	1.9 : 1			2.1 : 1			2.0 : 1		

Table III shows the results for amylase and β-glucuronidase. For the hydrolysis of starch the ratio of the activity of the gray matter to that of the white matter was of the same order as that for the common esterase and the

TABLE III

AMYLASE (μGM. GLUCOSE PER 100 MGM. WET WEIGHT PER HR.) ACTIVITY AND β-GLUCURONIDASE (μGM. PHENOLPHTHALEIN PER 100 MGM. WET WEIGHT PER HR.) ACTIVITY OF GRAY MATTER AND WHITE MATTER OF DOG BRAIN

	Amylase (0.14% starch)			Amylase (0.14% glycogen)			β-Glucuronidase		
	No.	Mean	S.E. of mean	No.	Mean	S.E. of mean	No.	Mean	S.E. of mean
Gray matter	4	12.5	±0.91	4	15.8	±0.80	8	5.21	±0.35
White matter	4	6.20	±0.44	4	5.12	±0.86	8	6.64	±0.90
Gray matter : white matter ratio	2.0 : 1			3.1 : 1			0.78 : 1		

phosphomonoesterases, whereas for the hydrolysis of glycogen the ratio was somewhat higher. The β-glucuronidase was the only enzyme for which, on a wet weight basis, the activity of white matter exceeded that of the gray matter.

It is well known that the wet weight to dry weight ratio of gray matter is much greater than that of white matter (Johnson, McNabb, and Rossiter (19), for references). For the dog brain the mean wet weight to dry weight ratio was found to be 5.37 : 1 for gray matter, whereas for white matter the ratio was 3.24 : 1. If the results are expressed in terms of dry weight, the differences between the enzyme concentrations in gray matter and those in white matter become more obvious. Table IV gives the summary of the results expressed in this manner.

TABLE IV

ENZYME ACTIVITY OF GRAY MATTER AND WHITE MATTER OF DOG BRAIN, EXPRESSED IN TERMS OF DRY WEIGHT

Enzyme	Substrate	Substrate conc.	Gray matter	White matter	Gray matter : white matter ratio
Cholinesterase	Acetylcholine	0.006 *M*	22.1*	9.9*	2.1 : 1
	Acetyl-β-methylcholine	0.03 *M*	16.6*	6.5*	2.6 : 1
	Benzoylcholine	0.006 *M*	5.3*	1.9*	2.8 : 1
Common esterase	Tributyrin	0.007 *M*	24.4*	7.8*	3.1 : 1
Acid phosphomonoesterase	Disodium phenyl phosphate	0.004 *M*	2.58**	0.75**	3.4 : 1
Alkaline phosphomonoesterase	Disodium phenyl phosphate	0.004 *M*	10.6**	3.3**	3.2 : 1
Amylase	Soluble starch	0.14%	67.1†	20.1†	3.3 : 1
	Glycogen	0.14%	84.9†	16.6†	5.1 : 1
β-Glucuronidase	Phenolphthalein mono-β-glucuronide	0.001 *M*	28.0††	21.5††	1.3 : 1

* *ml. 0.001 N acid per 100 mgm. dry weight per hr.*
** *mgm. phenol per 100 mgm. dry weight per hr.*
† *μgm. glucose per 100 mgm. dry weight per hr.*
†† *μgm. phenolphthalein per 100 mgm. dry weight per hr.*

Discussion

Cholinesterase

Nachmansohn (27) found that the gray matter of dog spinal cord contained a greater cholinesterase activity than did the white matter and subsequently Little (22) reported that the gray matter from several different parts of dog brain contained a greater cholinesterase activity than white matter. The difference between the cholinesterase activity of the gray matter and that of the white matter reported here for dog brain is less than would be anticipated from the results of the above workers. However, it is now known that the concentration of cholinesterase in gray matter from different regions of the central nervous system may differ greatly (22, 28).

It is generally stated that cholinesterase of the central nervous system is exclusively of the 'true' or 'specific' type (23, 25, 29). The experiments reported here, like those of Sawyer and Everett (36), would indicate that, although true cholinesterase is the principal cholinesterase of the brain, there is also a definite concentration of pseudocholinesterase. This may be associated with admixed connective tissue elements, for peripheral nerve, which contains a much greater proportion of connective tissue, also contains a much greater concentration of pseudocholinesterase (4, 14, 35).

Common Esterase

Previous reports on the distribution of common esterase between gray matter and white matter of brain are conflicting. Quinan (32) found that the gray matter of human brain hydrolyzed ethyl butyrate more rapidly than the white matter and English and MacArthur (10) reported similar results using monoacetin as substrate. On the other hand, Takasaka (39) found that the white matter of human brain hydrolyzed tributyrin more rapidly than the gray matter. However, none of these reports gives a true indication of the distribution of common esterase, for in no instance was the activity of the cholinesterases inhibited and, of the substrates used, ethyl butyrate and tributyrin are known to be readily hydrolyzed by pseudocholinesterase (Adams and Whittaker (2), for references) and monoacetin is known to be readily hydrolyzed by true cholinesterase (Adams (1), for references). In the experiments reported here, in which the activity of the cholinesterases was inhibited by eserine, the activity of common esterase was definitely greater in gray matter than in white matter.

Phosphomonoesterases

The observation that in both gray matter and white matter the activity of alkaline phosphomonoesterase exceeded that of acid phosphomonoesterase is in agreement with the reports of Edlbacher, Goldschmidt, and Schlappi (9) and Fleischhacker (12) for ox brain. However, for alkaline phosphomonoesterase, Edlbacher *et al.* (9) found that the activity in white matter exceeded that in gray matter, whereas Fleischhacker (12) found that the activity in gray matter, exceeded that in white matter. In the work reported here on dog brain there was no doubt that the activity of both acid and alkaline phosphomonoesterase in gray matter greatly exceeded that in white matter.

Amylase

Takasaka (39) reported that the amylase activity of the gray matter of human brain was about twice the activity found in white matter, a result very similar to that reported here for dog brain.

β-Glucuronidase

As far as is known, no reports have appeared on the presence of β-glucuronidase in brain tissue. Although the concentration of β-glucuronidase in dog brain was found to be considerably less than that reported for most other

tissues (40), the enzyme was certainly present. The concentration was greater than could be explained by the β-glucuronidase activity of incorporated blood or tissue fluid. β-Glucuronidase was the only enzyme for which the activity in white matter exceeded that in the gray matter.

General

The separation of gray matter from white matter in dog brain is at best only an approximate procedure. The gray matter in particular was contaminated with traces of white matter. It should also be remembered that the concentration of a certain enzyme in the gray matter from one area of the brain may differ from that in another area (22, 28) and, even within the cerebral cortex of the same area, the concentration of some enzymes may vary greatly with the different cytoarchitectural layers (31). Thus the data presented here show, only in a very relative way, the distribution of enzymes between gray matter and white matter of dog brain.

For all the enzymes studied, with the exception of the β-glucuronidase, the activity in the gray matter was greater than that in the white matter, although the relative activities differed for each enzyme. This was to be expected, since the gray matter contains many nerve cell bodies and the white matter is rich in myelin material, which is usually considered to be inert. The finding of a higher β-glucuronidase activity in white matter than in gray matter raises the problem of the origin of this β-glucuronidase. Apart from the neurones, the central nervous system also contains microglia, oligodendrocytes, and astrocytes. To determine whether any of these cell types occur in greater numbers in the white matter of dog brain than in the gray matter would form the subject of an interesting complementary cytological research. In this connection it should also be remembered that the astrocytes are of two types: the cytoplasmic astrocyte, which is predominantly in the gray matter, and the fibrous astrocyte, which is predominantly in the white matter.

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POLIOMYELITIS IN CANADIAN ESKIMOS

LABORATORY STUDIES. II.[1]

By Eina M. Clark and A. J. Rhodes

Abstract

This paper describes further laboratory studies undertaken to investigate the immunological aspects of the outbreak of poliomyelitis in the Canadian Eastern Arctic in the winter of 1948–1949. No evidence was found that any of three strains of virus recovered from Eskimos was pathogenic for cotton rats or mice. No strains of Coxsackie virus were recovered. Main attention is directed to the examination of 51 sera from 40 persons (34 Eskimos) involved in the outbreak for the presence of neutralizing antibody to Lansing poliomyelitis virus. Antibody was found in the acute phase serum of two of three patients, and in the convalescent phase serum of about half of those tested. The serum of six of eight healthy Eskimos and five of six white contacts contained antibody. It seemed that antibody started to appear in the serum from the age of 10 onwards, the serum of most adults proving positive. It is concluded that the presence of Lansing antibody in the serum of the Eskimos was the result of previous exposure to Lansing virus and was unrelated to the current epidemic. The world-wide prevalence of Lansing antibody and by inference of Lansing virus is evident.

Eskimos living in the Eastern Arctic region of Canada were involved in a severe outbreak of poliomyelitis in the winter of 1948–1949. The clinical and epidemiological details of this epidemic have already been fully reported (1, 24), and the isolation in our laboratory of poliomyelitis virus from several representative patients at Chesterfield Inlet, the area mainly affected, has been described (26). It remained however to investigate some of the immunological aspects of this outbreak. In particular, information was required relating to the biological and antigenic characteristics of the causal strain (or strains) of poliomyelitis virus, and concerning the degree of previous exposure of the population to poliomyelitis. This paper records the results of three lines of investigation undertaken with these objects in view.

First, attempts were made to determine whether the causal strain was pathogenic for cotton rats and mice, for it appears that rodent pathogenicity is a feature of the Lansing type of poliomyelitis virus (6).*

Second, tests were performed to see whether Coxsackie and poliomyelitis viruses were both present in stools (18, 27).

Third, and this constituted the major part of the investigation, tests for Lansing virus neutralizing antibody were carried out on the sera of 40 persons, 34 of whom were Eskimos. This test is now regarded as a valuable tool in

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Contribution from the Connaught Medical Research Laboratories, with the collaboration of the Laboratory of Hygiene, Department of National Health and Welfare, Ottawa.

* *A strain of poliomyelitis virus isolated in our laboratory from Eskimo No. E3–213 was sent to Doctor David Bodian for typing. He has found strong evidence that this patient was infected with the Brunhilde type of virus, the type commonly found on the North American Continent. Virus isolated from another patient (No. E3–177) is now under examination by Doctors John Kessel and Charles Pait.*

the study of the epidemiology and immunology of poliomyelitis (9, 11, 17, 23, 28, 31). It has been found for example that under conditions of poor hygiene, Lansing antibody is commonly acquired early in life, in the tropics even by two years of age, and correspondingly under better hygienic conditions antibody is acquired later in childhood (11, 17, 28, 31). These differences have been explained on the basis of a heavier exposure to poliomyelitis virus in communities where hygiene is poor, than in groups where living standards are higher.

Although this test has been used as an epidemiological tool, the status of Lansing neutralizing antibody itself is not by any means clear. The antibody may, as many believe, represent a specific immune body resulting from infection with Lansing type poliomyelitis virus. If this is so, it is perhaps unexpected that comparatively few strains of Lansing type virus have so far been recovered. No worker yet seems to have tried to correlate in any given community the incidence of persons whose serum contains Lansing antibody with the incidence of persons excreting Lansing virus.

An alternative explanation for the presence of Lansing antibody is that it may develop as the result of infection with another type of poliomyelitis virus, but experimental work does not support this suggestion.

Thirdly, it is possible that the Lansing antibody present in the serum of the majority of older persons and many children has not arisen as a result of infection with poliomyelitis virus, but that it represents antibody developed to some benign agent, presumably viral, commonly present in the human body. No such agent has ever been discovered. It is possible that some refined serological technique might demonstrate a difference between the Lansing antibody of "normal" adult serum and the Lansing antibody that develops in convalescence from infection with a Lansing type of virus.

It seems to us that the first of these explanations is, as far as can be judged at the moment, the most credible. Accordingly, it was held that the results of the Lansing antibody test would serve to indicate the degree of previous exposure of this group of Eskimos to Lansing type poliomyelitis virus. Evidence of previous exposure to Lansing type poliomyelitis virus would presumably indicate that the community might also have been exposed to other types of poliomyelitis virus. By the performance of the test, some indirect evidence concerning the immune status of the Eskimos might thus be obtained.

Rather unexpectedly we found Lansing antibody present in the serum of 18 of the 34 Eskimos studied. Antibody was demonstrated in the serum of 1/8 children aged 5 to 9, 5/5 aged 10 to 14, 2/8 aged 15–19, and 10/13 adults aged 20 and over. Here it is of interest to mention that a serological survey of Alaskan Eskimos has recently been reported by Paul and Riordan (23). They found very few positive sera from persons under the age of 20, whereas the serum of practically all persons over this age contained antibody. It would appear therefore that the Canadian Arctic Eskimos show evidence of a higher degree of exposure to Lansing virus.

Additional observations that we have made suggest that under certain conditions Lansing antibody may persist in the circulating blood serum for a relatively short period.

Characteristics of the Poliomyelitis Outbreaks in Eskimos, Winter of 1948-1949

The essential features of the two interconnected outbreaks of poliomyelitis in the Eastern Arctic may be briefly reviewed (1, 24). The area involved lies on the northwest coast of Hudson Bay, in the Northwest Territories and north of the port of Churchill, Man. For administrative purposes, the area is divided into the more southerly area E1 and E3 further north. Area E1 contains about 500 Eskimos trading into various posts, and in area E3, including the settlement of Chesterfield Inlet, there are about 275 Eskimos. There is said to be little if any contact in the winter time between Eskimos in the two areas.

The original source of infection of these areas can probably be traced back to Churchill, where there is known to have been a case of poliomyelitis in the summer of 1948. However, first involved in the outbreaks studied was the E1 area which includes the posts of Nunella, Eskimo Point, Padlei, and Kazan. Cases of poliomyelitis occurred in this area from September 1948 to March 1949. Of the 500 Eskimos in the area, 21 became paralyzed, and there were four deaths. The approximate attack rate was therefore 5%, an exceptionally high figure. In addition to these cases in Eskimos, one white missionary developed paralysis. The infection next spread northward to Chesterfield Inlet where an "explosive" outbreak occurred between February 14 and March 7, 1949. Of the 275 Eskimos in the settlement and immediate vicinity 39 were paralyzed and another 14 died; the paralytic attack rate was therefore about 14%, and the percentage of Eskimos attacked by the disease was about 20%. This high attack rate was attributed to increased opportunities for infection occasioned by a heavy congregation of Eskimos in and near the settlement, whereas in the more southerly area the Eskimos were dispersed over a wide area. None of six white children at Chesterfield developed paralysis, but four of these, along with two of the 19 white adults suffered from a probable attack of abortive poliomyelitis. At Chesterfield, there were no cases in Eskimos under three years of age, but otherwise the incidence in the various age groups was relatively even. It is apparent that the Eskimos at Chesterfield Inlet and in the E1 area were highly susceptible to paralytic poliomyelitis. The exposed white persons at Chesterfield escaped paralysis, but probably not infection with the virus. The epidemiological evidence suggests that infection was transmitted by healthy and convalescent carriers, and that a single strain was probably involved in the E1 and E3 outbreaks.

We have been informed by Dr. W. J. Wood of the Department of National Health and Welfare, Indian Health Services, that only a few more cases of poliomyelitis occurred in the affected areas after the period covered by the reports cited.

Previous Exposure of Eskimos to Poliomyelitis*

All Eskimos in the areas concerned have had contacts from infancy with white men such as traders, missionaries, police, radio operators, and boat and plane crews. These contacts would be less numerous in the case of Eskimos living in the interior districts such as Padlei and Kazan. Some at least of the Eskimos have had direct or indirect contacts with the port of Churchill. As regards contacts one with another, the communal life and hygienic standards of the Eskimo are such as definitely to facilitate transmission of any diseases in which the causal agent is present in nasopharynx and stool.

With the opportunities that must have arisen under the above conditions for the occasional introduction of poliomyelitis, it is perhaps unexpected to learn that there are no prior records of this disease in the Eastern Arctic. No Eskimos have been found with residual paralysis. A police sergeant at Chesterfield Inlet, with at least 15 years experience in the Eastern Arctic did not recall any outbreaks of paralytic illness. The police records of deaths and epidemics at Chesterfield Inlet did not suggest that poliomyelitis had occurred before.

In fact, there have been only a few published accounts of poliomyelitis in Eskimos in other parts of the Arctic. For example, outbreaks have occurred in Greenland (13, 14). Of particular interest is the paper of Paul and Riordan (23) which reports the occurrence of poliomyelitis on the North Coast of Alaska about 20 years ago, but apparently not more recently.

Laboratory Investigations

I. Attempts at Propagation of Chesterfield Inlet Strains of Poliomyelitis Virus in Rodents

These investigations were carried out some time after our original work in which poliomyelitis virus was recovered from several Eskimos affected with poliomyelitis at Chesterfield Inlet (26). Pathological material from three patients previously shown to contain poliomyelitis virus was selected (E3–56, E3–177, E3–227). The materials were suitably prepared, and inoculated cerebrally into adult mice, cotton rats, and monkeys. All three monkeys developed poliomyelitis, indicating the presence of virus in the inoculum. The mice and the cotton rats remained healthy and after two to three weeks were sacrificed and their brains and cords removed. Suspensions were prepared and passaged further through mice and cotton rats. In all, three passages were carried out in mice and three in cotton rats by this technique of "blind passage". In no case, did any animal show any sign suggestive of the presence of a rodent-pathogenic neurotropic virus. The surviving cotton rats became paralyzed when challenged with Lansing virus. Further,

* *For much of the information in this section we are indebted to Dr. W. J. Wood, Indian Health Services, Winnipeg.*

nervous tissue from each of the three positive monkeys was inoculated in mice and cotton rats with negative results. Fuller details of these experiments are shown in Table I.

TABLE I

ATTEMPTS AT PROPAGATION OF CHESTERFIELD INLET STRAINS OF POLIOMYELITIS VIRUS IN RODENTS

Patient's reference number	Pathological specimen examined and method of preparation	First passage experiments	Subsequent passage experiments
E3–56	Brain, 20% suspension	Monkey #781, positive	Tissue from #781 inoculated in mice and cotton rats, with negative results
		Adult mice and cotton rats unaffected	Two further passages carried out at 2–3 week intervals, mouse to mouse, cotton rat to cotton rat, were negative*
E3–177	Stool, deposit from ultracentrifugation	Monkey #783, positive for poliomyelitis	Tissue from #783 inoculated in mice and cotton rats with negative results
		Adult mice and cotton rats unaffected	Two further passages were negative*
E3–227	Throat washings, treated with antibiotics	Monkey #785 positive for poliomyelitis	Tissue from #785 inoculated in mice and cotton rats with negative results
		Adult mice and cotton rats unaffected	Two further passages were negative*

* *All surviving cotton rats were challenged cerebrally with Lansing virus mouse pool (30 mouse* LD_{50}*); within eight days all rats became paralyzed and died.*

It is concluded that the three strains of virus examined are not pathogenic for rodents.

II. EXAMINATIONS FOR COXSACKIE VIRUS

Poliomyelitis and Coxsackie viruses may be found together in a single sample of stool. Accordingly, a number of pathological materials from Eskimos were tested in suckling mice to determine whether any Coxsackie virus was present. The results of these tests, along with those for poliomyelitis virus by monkey inoculation are shown in Table II. In both cases diagnosis was made by histological examination of affected animals. It will be seen that no Coxsackie virus was isolated from any of seven patients, whereas poliomyelitis virus was recovered from six of those tested. We have not ourselves yet made extensive attempts to passage these strains, but it can be stated that several monkeys inoculated with the nervous tissue of first passage-monkeys have failed to become paralyzed.

TABLE II

RESULTS OF INOCULATION OF PATHOLOGICAL MATERIALS IN SUCKLING MICE

Reference number of Eskimo	Pathological material	Method of preparation of inoculum	Result of inoculation of animals	
			Suckling mice for Coxsackie virus	Rhesus monkeys for poliomyelitis virus
E3–56	Brain and cord	20% suspension	–	+
E3–164	Brain and cord	20% suspension	–	+
E3–55	Stool	Ultracentrifugation	–	–
E3–227	Stool	Ultracentrifugation	–	Not tested
E3–227	Throat washings	Antibiotics	–	+
E3–79	Stool	Ultracentrifugation	–	+
E3–177	Stool	Ultracentrifugation	–	+
E3–213	Stool	Ultracentrifugation	–	+
E3–213	Throat washings	Antibiotics	–	Not tested

III. EXAMINATIONS FOR LANSING VIRUS NEUTRALIZING ANTIBODY

Technique

In general, the technique is similar to that used by workers at the Johns Hopkins University, Baltimore (3, 4, 19, 31, 32, 33, 34). The main principle is that undiluted serum and serial tenfold dilutions thereof are mixed with a fixed amount of Lansing virus. After a period of contact, the mixtures are inoculated intracerebrally in mice. After observation of the mice for five weeks, the 50% neutralizing (protective) capacity of the serum is calculated.

Storage of Sera

The sera that we have tested were received in three main shipments. Sera taken at the time of the Chesterfield outbreak in 1949 were flown packed in dry ice to Ottawa and delivered to the Laboratory of Hygiene, where they were held frozen in an electric deep-freeze at about −28° C. These sera were shipped to us in the frozen state about a year later (February, 1950). Other batches of sera were taken in the hospital in Winnipeg to which several of the Eskimos were admitted for treatment. These sera were taken in March, 1949, and again in March, 1950, and shipped to us immediately. All sera were held by us in a carbon dioxide ice chest pending testing. It is unlikely that during the period of 12–15 months storage these sera would have acquired any capacity for nonspecific virus neutralization (21). Nor is it likely that during this period there was a significant drop in any Lansing antibody present in the original specimens (34).

All sera used in these studies were unheated and free from preservative.

Preparation of Serum Dilutions

Tenfold dilutions of the sera to be tested were prepared in sterile physiological saline and held at 4° C. overnight, to save time during the actual performance of the test the following day. When several samples from one patient taken at different intervals after onset were available for test, all were included in the same batch to facilitate accurate comparison of antibody levels.

Pool of Lansing Virus

In practically all of the tests here reported, the virus used was one known to us as "Lansing mouse pool No. 1". This pool was prepared from the spinal cords of 97 mice harvested between the second and 12th days after the cerebral inoculation of various dilutions of mouse nervous tissue infected with Lansing virus. It was filled into small screw-capped vials as a 20% suspension in saline and stored in the carbon dioxide ice chest. The virus was originally sent to us by Dr. Howard Howe. This mouse pool No. 1 has been used by us during the past year for large numbers of neutralization tests with human and animal immune sera, and has proved satisfactory.

During the conduct of these and other estimations of Lansing antibody titers, the pool was titrated on numerous occasions by the cerebral inoculation (0.03 ml.) of suitable dilutions of pool, usually from $10^{-2.7}$ to $10^{-4.7}$, in groups of 8 or 10 male mice, 14 to 16 gm. weight. The mice were observed for a period of four weeks after inoculation and deaths were recorded daily. The LD_{50} value of the virus suspension used in each titration was determined by plotting the probits of the percentage mortality of the mice in each group against the corresponding logarithm of the virus dilution inoculated, drawing a straight line between the points, and reading off the probable dilution at which 50% mortality might be expected. This method gives results in close agreement with those obtained by the procedure of Reed and Muench (25).

In order to arrive at an estimation of the cumulative LD_{50} titer of this mouse pool, a similar calculation was made using the cumulative mortalities in the total number of mice inoculated with any given dilution of pool. These figures were as follows:

Dilution of mouse pool #1	Total number of mouse deaths	Cumulative mortality as percentage
$10^{-2.2}$	39/41	95
$10^{-2.7}$	376/425	88.6
$10^{-3.2}$	405/574	70.6
$10^{-3.7}$	356/584	61
$10^{-4.2}$	159/362	44.0
$10^{-4.7}$	76/248	30.6

The cumulative LD_{50} value of mouse pool No. 1 calculated from the above figures was $10^{-4.03}$.

Preparation of Virus–Serum Mixtures

To one volume of undiluted serum and to one volume of serial tenfold dilutions thereof was added one volume of pool suspension, diluted so that the final dilution of pool in each tube of virus–serum mixture was $10^{-2.7}$. As the cumulative LD_{50} value was $10^{-4.09}$ the dose of virus inoculated into each mouse was on the average, 21 LD_{50}.

The virus–serum mixtures were left at room temperature for one to one and one-half hours before inoculation into mice.

Inoculation of Mice

After the virus–serum mixtures had stood at room temperature, groups of eight male mice were inoculated intracerebrally (0.03 ml. volumes). For the majority of the tests here reported the mice used were 14 to 16 gm. in weight, but for some tests animals of 12 to 14 gm. weight were used. The mice were observed for a period of five weeks. Deaths were recorded daily and survivors were counted at the end of the experiment. Deaths occurring on the day following inoculation were disregarded.

Controls

The average amount of virus inoculated in each mouse was, as stated, 21 LD_{50}. This figure was calculated from the cumulative mortalities in several hundred mice. It is not to be expected in a biological test involving so many uncontrollable variables that single titrations of virus suspension involving less than 50 mice would regularly give exactly this LD_{50} value. The majority of such single titrations would fall close to the average but some would be considerably lower and some considerably higher. It is certain that the use of too large an amount of virus will mask smaller amounts of antibody. Accordingly in each batch of neutralization tests, usually involving 400–500 mice, a complete titration of the virus suspension added to the virus–serum mixtures was performed. These titrations indicated that the LD_{50} of the virus suspension usually fell between 20 and 45 LD_{50}, a variation not sufficiently great to influence the antibody titers determined. However, on a few days values as high as 100 LD_{50} were obtained, and tests performed on such days were repeated.

Expression of Results

With the dilution of virus pool used ($10^{-2.7}$) the mortality rate in mice inoculated cerebrally was about 90% (see above). This means that in groups of eight mice receiving a mixture of virus and serum without antibody the number of deaths would usually be 8/8 or 7/8. Sometimes, however, by the operation of chance as few as six, five, or even four might die. Bearing this in mind, we have adopted rather rigorous criteria in the expression of our results, with the object of not classifying as positive any serum about which the result was in doubt. Our results have been classified on the following basis:

A "negative" serum is one where the mortality picture in mice inoculated with the virus-undiluted serum mixture was 8/8, 7/8, 6/8, 5/8, 4/8, or 3/8.

The term "positive", without qualification, is used for a serum where the mortality picture in mice inoculated with the virus-undiluted serum mixture was 0/8, 1/8, or 2/8. In such "positive" sera as a rule there was little evidence of protection in the mice inoculated with virus-diluted serum mixtures.

Thirdly, in more strongly positive sera, the degree of positivity is expressed in terms of the 50% neutralizing titer. These titers are calculated in the same way as virus titers. For example, a serum might be reported as showing a 50% neutralizing titer of $10^{-2.0}$. This would mean that on the average 50% of mice were protected from death when inoculated with 21 LD_{50} of virus and serum diluted 1 : 100. In calculating the 50% neutralizing titer of a serum, the fact that the serum is actually diluted with an equal volume of virus suspension is ignored. The 50% neutralizing titer refers therefore to the dilution of serum before addition of virus, and not to the final dilution of serum. The lowest 50% protective titer calculated is about $10^{-0.9}$.

As is inevitable in a test of this type, it has proved difficult to assess the results of some titrations. In particular, some sera have given "flat" responses, where 50% or so of mice have been protected in two or more successive dilutions. Although this type of response may be expected by chance, it appears to be characteristic of certain sera, and the significance is not at present apparent. Similar results have been obtained on repetition in some cases. Provisionally, such sera are reported as negative.

Repetition of Tests

In a few cases sera have been retested. The results of these repeat tests are shown in the various tables in brackets under the figures obtained in the original test. With regard to the reproducibility of any particular titration, we may quote the observations of Morgan (19) who found that there might be a fivefold (0.32 log) difference between the highest and lowest estimations of neutralizing end points in different determinations with a single sample. In the studies of Bell (4), it was found that a 50% protective end point obtained in any one titration, using 10-fold dilutions of a single serum sample, was accurate within a range of 0.4 log, i.e., the true value of any figure obtained would lie within 0.2 log above and below the end point determined.

Results

Some 51 serum samples from 40 persons have been investigated in this study. Except where stated otherwise, all were residents of the Chesterfield Inlet (E3) area; a few tests were performed on residents of the E1 area. Out of the 40 persons, 34 were Eskimos; 26 of these were clinically affected by poliomyelitis and eight were classified as healthy family contacts. Six white persons were also examined, five being healthy and one having had probable abortive poliomyelitis.

First will be presented the results of examining two or more samples from a single patient, then the results of examining various individual specimens of serum. Finally the results will be analyzed according to age groups.

Serum Antibody Levels in Paired Sera (Acute and Convalescent Phases)

Only five samples of serum obtained in the acute phase of illness were available for study. The earliest was obtained two days after onset, and the latest 10 days. The results shown in Table III can be summarized as follows:

Lansing antibody present in acute phase serum, two cases (titers $10^{-0.94}$, $10^{-0.9}$); Lansing antibody absent, two cases; result questionable, probably negative, one case. Convalescent phase serum was available from the two patients in whom antibody was present in the acute phase sample. In one case (Irkroa) the level fell from $10^{-0.9}$ to zero in the period of eight weeks between samplings; this difference in titer was probably significant. In the case of the other (Tudlik) the titer was not significantly different in the specimen taken four days after onset ($10^{-0.94}$), and in the specimen taken four weeks after onset ($10^{-0.9}$). The sera from the third patient (Karlik) were negative in convalescence. This patient was hospitalized in Winnipeg from shortly after the onset of illness for at least a year.

Serum Antibody Levels in Paired Sera (Early and Late Convalescent Phases)

Blood samples taken early and late in convalescence were available from six paralyzed patients hospitalized for at least a year in Winnipeg. The early samples were taken three-four weeks after onset (shortly after the patients' arrival in Winnipeg) and again 12 months after onset. The results of these tests are given in Table IV, and may be summarized as follows: Positive early convalescence, negative later, 3; positive early, lesser degree of positivity later, 1; negative at both times, 2.

It will be seen that there was an apparent drop in antibody level between three to four weeks and 12 months after onset in all of the four persons whose serum was positive in the early convalescent phase. It should be noted, however, that in the case of A. Suslutor the evidence for a definite fall in titer is questionable. The evidence for a fall in titer in the other three patients is more conclusive.

Serum Antibody Levels in Single Samples of Serum (Convalescent Phase)

Single samples were received from 14 patients. These sera had been removed either early in convalescence (4–5 weeks), in mid-convalescence (8–9 weeks), or late in convalescence (6–18 months). Two late convalescent phase sera were obtained from another patient. The results are given in Table V. The serum of six of these patients was positive and of nine was negative for Lansing antibody. It is of more interest, however, to consider these results along with those of the examinations of the other convalescent

TABLE III

LANSING ANTIBODY LEVELS IN PAIRED SERA TAKEN IN ACUTE AND CONVALESCENT PHASE, ESKIMO PATIENTS

Name and reference number	Age and sex	Clinical diagnosis	Samples withdrawn at	Date of onset of illness	Collection of serum		Mortality in mice receiving fixed dose of virus (21 LD_{50}) and following dilutions of serum				50% Neutralization point of serum or other expression of activity
					Date	Period after onset	Undil.	$10^{-1.0}$	$10^{-2.0}$	$10^{-3.0}$	
Mablik, A. (E3–981)	8,F	NPP*	CI†	17 Apr., '49	27 Apr., '49	10 days	5/8	7/8	7/8		Negative
Tudlik, T. (E3–215)	11,M	P*	CI	20 Feb., '49	24 Feb., '49	4 days	2/8	3/8	8/8		$10^{-0.94}$
			W†		22 Mar.,'49	4 weeks	1/8	5/8	8/8		$10^{-0.9}$
Irkroa, M. (E3-57)	11,F	P	CI	18 Feb., '49	24 Feb., '49	6 days	1/8	5/8	7/8		$10^{-0.9}$
			CI		27 Apr., '49	9 weeks	8/8	8/8	8/8		Negative
Karlik, P. (E3–145)	18,M	P	CI	20 Feb., '49	22 Feb., '49	2 days	3/8	4/7 (5/8)	4/8 (9/9)		All probably negative
			W		22 Mar., '49	4 weeks	4/8	5/8 (9/10)	4/8 (10/10)	(9/10)	
			W		27 Feb., '50	12 months	6/8	6/8	5/8	7/8	
Tautu, S. (E3–56)‡	18,M	P, fatal	CI	20 Feb., '49	25 Feb., '49 (day of death)	5 days	7/8 (tested 1 : 3 dilution)	8/8	7/8		Negative

* *P, paralytic; NPP, nonparalytic, poliomyelitis.*

† *CI, Chesterfield Inlet; W, Winnipeg.*

‡ *Poliomyelitis virus isolated from pathological material.*

TABLE IV

LANSING ANTIBODY LEVELS, EARLY COMPARED WITH LATE CONVALESCENT PHASE SERA, PARALYTIC ESKIMO PATIENTS

Name and reference number	Age and sex	Date of onset of illness	Collection of serum		Mortality in mice receiving fixed dose of virus (21 LD_{50}) and following dilutions of serum				50% Neutralization point of serum or other expression of activity
			Date	Period after onset	Undil.	$10^{-1.0}$	$10^{-2.0}$	$10^{-3.0}$	
Tinnuadluk, M. (E3-247)	9M,	17 Feb., '49	22 Mar., '49	4 weeks	4/8	4/8	8/8		Questionable negative (No significant change in titer)
			27 Feb., '50	12 months	4/8	7/8	7/8	7/8	
Aklunark, S. (E3-177)‡	11,M	24 Feb., '49	22 Mar., '49	4 weeks	0/8	3/8	6/8		$10^{-1.35}$
			27 Feb., '50	12 months	7/8	8/8	8/8	8/8	Negative
Sammurtok, T. (E3-30)	15,M	Feb., '49	22 Mar., '49	About 4 weeks	2/8	5/8	5/7		Positive
			27 Feb., '50	12 months	7/8	6/6	8/8	7/7	Negative
Suslutor, A. (E3-43)	15,M	19 Feb., '49	22 Mar., '49	4 weeks	2/8	8/8	7/8		Positive
			27 Feb., '50	12 months	4/8	7/8	6/7	8/8	Negative
Kolit, S. (E3-121)	32,M	Feb., '49	22 Mar., '49	About 4 weeks	5/8	5/8	8/8		Negative
			27 Feb., '50	12 months	7/8	8/8	7/8	8/8	Negative
Sammurtok, V. (E3-227)‡	46,M	2 Mar., '49	22 Mar., '49	3 weeks	0/6 (1/10)	1/7 (2/7) (0/7)	3/8 (5/10) (5/8)	(9/10) (6/8)	Over $10^{-2.0}$ ($10^{-1.7}$) ($10^{-2.25}$)
			27 Feb., '50	12 months	1/7	8/8	8/8	6/6	Positive

‡ *See footnote Table III.*

TABLE V

LANSING ANTIBODY LEVELS IN CONVALESCENT PHASE SERA, PARALYTIC ESKIMO PATIENTS*

Name and reference number	Age and sex	Date of onset of illness	Collection of serum		Mortality in mice receiving fixed dose of virus (21 LD_{50}) and following dilutions of serum				50% Neutralization point of serum, or other expression of activity
			Date	Period after onset	Undil.	$10^{-1.0}$	$10^{-2.0}$	$10^{-3.0}$	
A. *Samples removed 4–5 weeks after onset*									
Tanuuyark, G. (E3–199)	7,M	Feb., '49	22 Mar., '49	About 4 weeks	4/8	4/8	6/8		Probably negative
					(4/10)	(8/10)	(10/10)	(10/10)	Negative
Yerark, S. (7629)	7,M	17 Feb., '49	22 Mar., '49	5 weeks	5/8	5/8	8/8		Negative
Miluk, M. (11501)	8,F	16 Feb., '49	22 Mar., '49	5 weeks	7/8	6/8	8/8		Negative
Piktaosar, L. (E3–789)	14,F	20 Feb., '49	22 Mar., '49	4 weeks	1/8	6/8	8/8		Positive
B. *Samples removed 8–9 weeks after onset*									
Niakordluk, B. (E3–214)	15,F	Feb., '49	27 Apr., '49	About 8–9 weeks	5/8	5/8	6/8		Negative
					(8/9)	(9/9)	(9/10)	(9/10)	Negative
Puyuat, M-A. (E3–213)	18,F	26 Feb., '49	27 Apr., '49	8 weeks	8/8	8/8	8/8		Negative
C. *Samples removed 6–18 months after onset*									
Marcussi (from Tavane)	6,M	30 Apr., '49	27 Feb., '50	10 months	6/8	8/8	8/8		Negative
					(3/8)	(6/8)	(7/8)		Negative

* *All samples withdrawn in Winnipeg.*

TABLE V—*Concluded*

LANSING ANTIBODY LEVELS IN CONVALESCENT PHASE SERA, PARALYTIC ESKIMO PATIENTS*—*Concluded*

Name and reference number	Age and sex	Date of onset of illness	Collection of serum		Mortality in mice receiving fixed dose of virus (21 LD_{50}) and following dilutions of serum				50% Neutralization point of serum, or other expression of activity
			Date	Period after onset	Undil.	$10^{-1.0}$	$10^{-2.0}$	$10^{-3.0}$	
C. *Samples removed 6-18 months after onset*—Concluded									
Elungetah (E1–334) (from Padlei)	8,M	24 Dec., '48	27 Feb., '50	14 months	1/8 (1/8)	2/8 (5/8)	7/8 (6/8)	(6/7)	$10^{-1.3}$ Approx. $10^{-1.0}$
Alchuk (E1-17) (from Padlei)	15,M	Oct., '48	27 Feb., '50	16 months	7/8	6/8	6/8	8/8	Negative
Kanayor, S. (E3–149)	18,M	6 Mar., '49	27 Feb., '50	12 months	4/8	7/8	8/8		Negative
Howmik, Mrs. (from Padlei)	25,F	?	27 Feb., '50	Probably about 18 months	7/8	8/8	7/8	8/8	Negative
Gibbons, J. (E1–1)	30,M	Oct., '48	27 Apr., '49	About 6 months	3/8	6/8	6/8		Interpreted as positive
			27 Feb., '50	About 16 months	1/8	8/8	8/8	8/8	Positive
Pootanyank (E1–376) (from Padlei)	31,M	Dec., '48	27 Feb., '50	14 months	2/8 (2/8)	4/8 (6/8)	6/8 (6/8)	6/8	$10^{-1.15}$ Approx. $10^{-1.0}$
Parngnark (E1–104) (from Padlei)	39,M	Oct., '48	22 Apr., '49	About 6 months	2/8	8/8	7/8		Positive
Kubluitok, I. (E3–131)	40,M	May, '49	27 Feb., '50	9 months	0/8 (2/8)	8/8 (3/8)	8/8 (5/8)	8/8	Positive ($10^{-1.4}$)

* *All samples withdrawn in Winnipeg.*

phase sera shown in Tables III and IV. The combined results are as follows: Patients in whom serum was positive at some stage of convalescence, 11; patients in whom serum was negative in convalescence, 13. Thus Lansing antibody was detected at some period of convalescence in the sera of 45% of those tested.

These results can be expressed somewhat differently by analyzing the percentage of positive samples withdrawn at the various periods in convalescence (Table VI).

TABLE VI

DISTRIBUTION OF POSITIVE SERA ACCORDING TO PERIOD IN CONVALESCENCE

Period after onset when serum collected	Number and percentage of sera containing Lansing antibody
3–5 weeks	6/12 (50%)
8–9 weeks	0/3 (0)
6–18 months	6/16 (36%)
Any period	12/31 (40%)

It can be seen that positive samples were obtained in each of the three periods of convalescence.

Serum Antibody Levels in Healthy Eskimo Contacts

Blood was taken from eight Eskimo contacts, about two months after the outbreak at Chesterfield Inlet. As shown in Table VII, the serum of six of these persons contained antibody; the titers in five adults ranged from $10^{-1.0}$ to $10^{-2.0}$. These persons were all members of families having one or more cases of paralysis. Although the Eskimos in question gave no history of illness, it is not improbable that some or all of them had the abortive or "minor illness" type of the disease.

Serum Antibody Levels in Various Age Groups

In Table VIII are shown the results of antibody tests according to age group. The numbers in each group are too small for individual comparisons to be made, but it would appear that antibody starts to develop between the ages of five and nine and that the serum of most persons of 20 and over contains antibody.

Serum Antibody Levels in Healthy White Contacts

Blood was taken from six white adults employed at Chesterfield Inlet during the outbreak, the samples being withdrawn two months afterwards.

TABLE VII

LANSING ANTIBODY LEVELS, HEALTHY ESKIMO FAMILY CONTACTS, CHESTERFIELD INLET

Name and reference number	Age and sex	Date of collection of serum	Mortality in mice receiving fixed dose of virus (21 LD_{50}) and following dilutions of serum				50% Neutralization point of serum, or other expression of activity
			Undil.	$10^{-1.0}$	$10^{-2.0}$	$10^{-3.0}$	
Sulurayok, B. (E3–178)	7,M		8/8	8/8	7/8		Negative
Eguptak, (E3–232)	11,F		2/8	7/8	5/8		Positive
Uyaralak (E3–175)	31,F		0/8	1/8	4/8		Approx. $10^{-2.0}$
Pélagie, (E3–132)	35,F	27 Apr., '49 i.e. about 2 months after outbreak	2/8	0/8	8/8		$10^{-1.25}$
Ayaruak (E3–54)	42,M		3/8	6/8	8/8		Negative
Igalark, L. (E3–228)	46,F		0/8	4/8	8/8		$10^{-1.0}$
Putulik, P. (E3–144)	53,M		3/8 (0/9)	0/8 (3/9)	6/8 (8/9)	(9/9)	$10^{-1.5}$ ($10^{-1.25}$)
Atitak (E3–53)	59,F		0/8	4/8	7/8		$10^{-1.0}$

TABLE VIII

DISTRIBUTION OF POSITIVE SERA ACCORDING TO AGE GROUPS

Age group	Number of Eskimos with positive sera
5–9	1/8
10–14	5/5
15–19	2/8
20 and over	10/13
All age groups	18/34 (53%)

The results are given in Table IX from which it will be seen that the serum of only one person was negative, the titers in the others ranging from $10^{-1.35}$ to $10^{-2.6}$. Of these white adults, five gave no history of illness but one had a probable minor illness.

TABLE IX

LANSING ANTIBODY LEVELS, WHITE CONTACTS, CHESTERFIELD INLET

Name and reference number	Clinical features	Age and sex	Date of collection of serum	Mortality in mice receiving fixed dose of virus (21 LD_{50}) and following dilutions of serum				50% Neutralization point of serum, or other expression of activity
				Undil.	$10^{-1.0}$	$10^{-2.0}$	$10^{-3.0}$	
LB	Abortive polio.	25,M	27 Apr., '49 i.e. about 2 months after outbreak	6/8	8/8	7/8	7/8	Negative
MC	Nil	25,M		0/8	2/8	6/8	8/8	$10^{-1.5}$
Dr. M	Nil	30,M		0/8	3/8	6/8	7/8	$10^{-1.85}$
Brother B	Nil	40,M		0/8 (0/5)	3/8 (5/8)	6/8 (8/8)	5/8 (8/8)	Approx. $10^{-1.0}$ $10^{-0.75}$
Father C	Nil	45,M		0/8	1/8	0/8	5/8	$10^{-2.0}$
Sgt. H	Nil	55,M		2/8	0/8	8/8	7/8	$10^{-1.5}$

Discussion

This paper describes three lines of investigation undertaken with the object of throwing further light on the immunological background of an epidemic of poliomyelitis in the Canadian Eastern Arctic in the winter of 1948–1949. In particular, it was desired to obtain information relating to the probable antigenic type of the causal virus, and the degree of previous exposure of the Eskimo population to poliomyelitis, since the distribution of the cases relatively evenly in the various age groups (apart from the group 0–4 years) suggested that the Eskimos were encountering the infecting strain for the first time.

Little discussion is required concerning the first two investigations. No evidence was found that three strains of poliomyelitis virus recovered from Eskimos involved in the Chesterfield Inlet outbreak were pathogenic for rodents. Presumably, therefore, the causal virus of this epidemic did not belong to the Lansing type.* Further, it appeared that the infections at Chesterfield Inlet were associated solely with the presence of poliomyelitis virus, as no isolations of Coxsackie virus were made from several pathological specimens.

Before discussing our third line of investigation, namely the performance of tests for Lansing neutralizing antibody, it may be advisable to present briefly the findings of others who have examined the serum of patients suffering from poliomyelitis (2, 8, 9, 10, 22,32). It has been found for example that Lansing antibody is present in the serum in the acute phase of the illness in nearly 50% of patients. Comparison of acute and convalescent phase serum usually shows that the original antibody level remains constant, and that it is exceptional for a rise to be detected. Rather more frequently, in fact, observers

* *See earlier footnote.*

have noted a definite tendency for the antibody level to fall during the progress of convalescence. These various observations suggest that the presence of Lansing antibody in the serum of a patient suffering from poliomyelitis is usually incidental, and the result of a previous exposure to Lansing virus. In confirmation, is the finding in several reported series that the percentage of sera containing antibody increases with age, and therefore with opportunities for acquiring infection.

It is evident that our findings, even though they relate to comparatively few samples, as was inevitable from the circumstances of this unusual outbreak, are in close agreement with those previously reported. The serum of 18 of 34 Eskimos (53%) contained Lansing antibody, and the result of the test was unrelated to the stage in the illness at which the sample was taken. No rise in level of Lansing antibody could be demonstrated in the few patients from whom suitable samples of serum were available. On the contrary, we found that in a number of cases, titrations revealed a fall in Lansing antibody level during the progress of the disease. These observations were made in a series of six paralyzed patients removed to a Winnipeg hospital shortly after the onset of their illness and treated there for upwards of a year. It may be presumed that these patients had little opportunity for acquiring a fresh infection with a Lansing type virus during their stay in hospital. Four of these patients showed Lansing antibody in the early convalescent phase serum taken three to four weeks after onset. When samples obtained 12 months after onset were tested, however, no antibody could be found in the serum of three of the patients, and in the fourth, the antibody level was lower. We may also mention here comparisons of acute and convalescent phase serum of two other hospitalized patients (Irkroa and Tudlik). In the case of Tudlik, only four weeks separated the samples taken in the acute and early convalescent phases, and the antibody level was found to be similar in both. With Irkroa, however, about 8 weeks separated the taking of the acute and mid-convalescent phase samples, the antibody level falling to zero during this period.

These few observations afford some confirmation for the recent suggestion of Hammon that Lansing antibody levels tend to fall unless there is repeated antigenic stimulation (11).

Our results point to an acquisition of Lansing antibody in the Eskimo from about the age of 10 onward, the serum of the majority of the adults proving positive. It is of interest to note that the four persons in whom Lansing antibody fell to zero during the period of our observations were all relatively young, two being aged 11, and two 15. It would seem to be in keeping with current knowledge of the immunology of poliomyelitis to suggest that Lansing antibody persists in the serum for a relatively short time only following primary infection. As the natural process of immunization is repeated, antibody tends to become less transient and a sustained level results.

It is of interest now to compare our findings with those in the only parallel series of observations relating to Eskimos that has been published. Paul and

Riordan (23) reported a similar trend in the incidence of antibody in the various age groups of "healthy" Alaskan Eskimos. In their series, however, only a few sera from Eskimos under 20 neutralized the virus, whereas the serum of most persons over this age contained antibody. It is possible that the use of a larger challenge dose of Lansing virus in the neutralization test may explain the finding of relatively few positive sera in the younger groups in the Alaskan series. A recent personal communication from Dr. John H. Dingle reports a similar scarcity of positive tests for antibody in the serum of younger Eskimos living on Baffin Island (30). It would seem therefore that the Eskimos we have tested living in the Canadian Arctic regions E1 and E3 in the Hudson Bay area show more evidence of previous exposure to Lansing virus than do Alaskan Eskimos. Until we have carried out a serological survey of larger numbers of "healthy" Eskimos any conclusions in this regard must be tentative.

It may be, of course, that we have found a higher incidence of positive sera in the Canadian Eskimos because the samples were taken from patients in various stages of convalescence from poliomyelitis. This explanation seems unlikely, particularly as extensive studies with laboratory animals have not suggested that infection with non-Lansing types of poliomyelitis virus commonly stimulates significant amounts of Lansing antibody (7, 12, 15, 16, 20). Nevertheless, the question of whether infection with a non-Lansing type of virus stimulates Lansing antibody must be regarded as open. Especially as certain findings in the serological studies of Steigman and Sabin (29) with poliomyelitis patients could be explained on such a basis. Furthermore, it is known that infected monkeys show some cross-resistance to heterologous strains (5), suggesting some sharing of antigens between the different types.

Considering all the data available about this particular outbreak of poliomyelitis in the Canadian Arctic, it seems reasonable to conclude as follows: The outbreak was probably caused by a non-Lansing type of poliomyelitis virus, and infection with this virus did not stimulate the production of Lansing antibody. The Eskimo population shows evidence of being infected endemically with Lansing type virus, as serum antibody can be demonstrated from the age of about 10 onward. In some Eskimos it appeared that Lansing antibody levels tended to fall during a period of hospitalization away from their normal environment. The presence of Lansing virus infections in the Canadian Eskimo community concerned can be inferred.

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NOTE

A "Loop" Method for Counting Viable Bacteria or Bacteriophage

COMMENT ON A PAPER BY I. N. ASHESHOV AND F. C. HEAGY

Asheshov and Heagy (2) have recently described a "loop" method for counting microorganisms, apparently unaware that a similar method was described by Burri (3) in 1928 which has since found wide use as a rapid and simple method for counting bacteria.

In addition to employing a calibrated loop for measuring the inoculum, Burri found it simpler to spread the contents of the loop evenly over the surface of a well dried agar slant rather than a plate, recommending as an added refinement the previous drying of the agar to avoid confluent and spreading growths. After incubation, the colonies were counted and multiplied by the appropriate factor to obtain the viable count.

This procedure, known as the "Burri Smear–Culture Method" or the "Burri Slant Method" is especially valuable for field work, both on the farm and in dairy and food plants (11, 12, 13, 14, 15, 16) since a minimum of equipment and supplies is needed. It has been referred to in each edition of Standard Methods of Milk Analysis of the American Public Health Association (1) since 1934, as well as in the Laboratory Manual of the International Association of Milk Dealers (10). Myers and Pence (19) also adapted Burri's loop method to the measurement of thermoduric bacteria in laboratory-pasteurized milk. Various other workers (3, 4, 5, 6, 7, 8, 9, 16, 17, 18, 20, 21) have reported using Burri's method or modifications thereof. It is strange that the numerous references to the loop method which has been so widely used in dairy and food microbiology should have escaped the notice of Asheshov and Heagy.

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